N-ACYLPHOSPHORAMIDITES AND THEIR USE IN OLIGONUCLEOTIDE SYNTHESIS

TECHNICAL FIELD OF THE INVENTION

5 The present invention relates to the synthesis of oligonucleotides, and intermediates useful in the synthesis thereof.

BACKGROUND OF THE INVENTION

10 Since the development of efficient and reliable methods for automated synthesis of oligonucleotides, and early observations about the potential therapeutic application of oligonucleotides, there is a high demand for new oligonucleotide analogues. This demand is due to

15 the fact that natural oligonucleotides undergo very rapid nucleolytic degradation to monomeric nucleosides and nucleotides in biological fluids *in vitro* and/or *in vivo*.

The therapeutic application of oligonucleotides is based on the selective formation of hybrids between

20 antisense oligonucleotides and complementary nucleic acids, such as messenger RNAs (mRNAs). Such hybrids inhibit gene expression by blocking protein translation. Successful inhibition of gene expression, however, requires the antisense oligonucleotide to be nuclease

25 resistant so that it can be transported through biological membranes and can hybridize selectively to a target complementary nucleic acid, thereby actively blocking protein translation. Among the diverse oligonucleotide analogues that have been tested for

30 antisense activity, those bearing phosphorothioate internucleotide linkages are the most nuclease resistant and, therefore, are the most widely used.

Oligonucleotides bearing phosphorothioate internucleotide linkages are typically prepared by sulfurization of a phosphite precursor which, in effect, substitutes a sulfur atom for one of the non-bridging oxygen atoms normally present in phosphodiesters. This substitution results in a stereogenic center at the phosphorus atom. Unfortunately, the sulfurization of oligonucleotide phosphodiesters prepared by conventional methods results in the formation of complex mixtures of diastereomers, since the precursors are typically diastereomeric with respect to phosphorus. The stereochemistry of the phosphorus center, however, is important in imparting nucleolytic stability in the oligonucleotide. Structural studies suggest that chirality at the phosphorus center alters the thermodynamics of duplex formation and the pharmacokinetic profiles of therapeutic oligonucleotides. Thus, synthetic methods and intermediates that enable one to control the stereochemistry of such thioated oligonucleotides and to prepare particular thioated oligonucleotides in high stereochemical purity are highly desired. Such methods and intermediates would enable one to optimize the nucleolytic stability of phosphorothioate oligonucleotides. It is even more desirable to develop synthetic methods and intermediates that enable one to control the stereochemistry of tricoordinated, as well as thioated, oligonucleotides and to prepare them in high stereochemical purity.

The most commonly used synthetic method for the synthesis of thioated oligonucleotides is the phosphoramidite method with stepwise sulfurization (see,

e.g., U.S. Patent Nos. 4,415,732, 4,668,777, 4,973,679,
4,845,205, and 5,525,719). This method uses
tricoordinated phosphorus precursors that normally
produce products containing a mixture of different
5   thioated oligonucleotide stereoisomers. The lack of
stereoselectivity in the phosphoramidite process is
primarily due to the non-stereoselective and non-
stereospecific acid-catalyzed nucleophilic substitution
reaction, which is typically required to effect
10   substitution. Even when diastereomerically pure P-chiral
precursors are used, the coupling reaction proceeds with
full epimerization at phosphorus.

Attempts have been made to control the
stereochemistry of phosphorus in the synthesis of
15   oligonucleotides. One attempt is a recently developed
stereoselective method, which is drawn to the synthesis
of thioated oligonucleotides using tricoordinated
phosphorus precursors for acid-catalyzed nucleophilic
substitution reactions. However, this approach has
20   narrow applicability, in that it is limited to the
synthesis of very short oligomers, particularly because
each successive nucleoside coupling step occurs without
complete stereoselectivity.

Another attempt at controlling phosphorus
25   stereochemistry in oligonucleotide synthesis involves a
method for the stereospecific synthesis of thioated
oligonucleotides utilizing tetracoordinated phosphorus
precursors to accommodate base-catalyzed nucleophilic
substitutions. However, this approach also has limited
30   applicability because a different type of
tetracoordinated phosphorus precursor must be used to
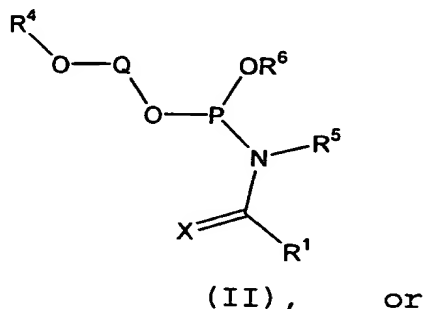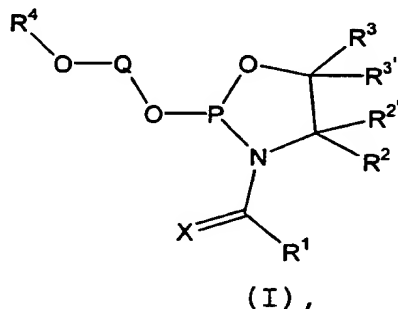
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generate a particular type of product, for example,
phosphates, phosphorothioates and phosphoroselenoates.
In other words, the structure of the desired product is
determined by the structure of the tetracoordinated

5   phosphorus precursor at the coupling step. Additionally,
separation of the diastereomers is difficult, and these
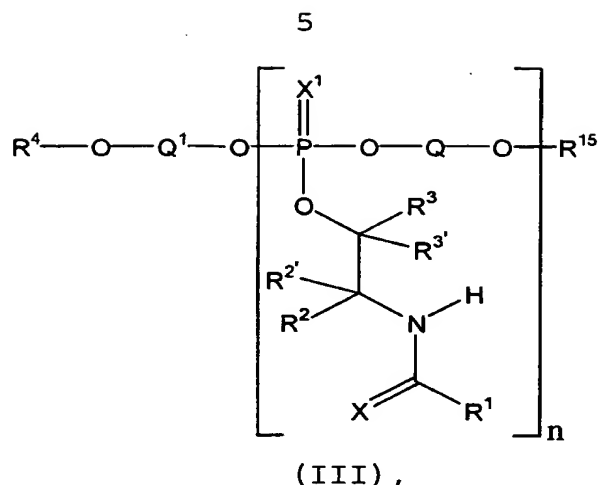tetracoordinated phosphorus precursors also are
hydrolytically unstable.

In view of the foregoing problems, there exists a

10   need for methods and intermediates that will permit the
efficient synthesis of unmodified or modified
oligonucleotides, particularly P-chiral oligonucleotides,
with high stereospecificity. The present invention
provides such methods and associated intermediates.

15   These and other advantages of the present invention, as
well as additional inventive features, will be apparent
from the description of the invention provided herein.

                     BRIEF SUMMARY OF THE INVENTION

20   The present invention provides a compound of the
formula:

(I),   (II),   or

5

$$R^4\!-\!O\!-\!Q^1\!-\!O\!\!\left[\!\!\begin{array}{c} X^1 \\ \| \\ P\!-\!O\!-\!Q\!-\!O \\ | \\ O \\ \end{array}\!\!\right]_n\!\!\!-\!R^{15}$$

(III),

wherein R^1 is an alkyl, an alkenyl, an alkynyl, a cycloalkyl, an aryl, or an aralkyl, wherein R^1 is

5   unsubstituted or substituted. R^2 and $R^{2'}$ are the same or different and each is H, an alkyl, an alkenyl, an alkynyl, a cycloalkyl, an aryl, or an aralkyl, wherein R^2 and/or $R^{2'}$ is unsubstituted or substituted. R^3 and $R^{3'}$ are the same or different and each is H, an alkyl, an

10   alkenyl, an alkynyl, a cycloalkyl, an aryl, or an aralkyl, wherein R^3 and/or $R^{3'}$ is unsubstituted or substituted. Alternatively, either of R^2 or $R^{2'}$, in combination with either of R^3 or $R^{3'}$, together with the carbon atoms to which they are bonded, form a cyclic

15   substituent, which can be unsubstituted or substituted. R^4 is a protecting group or a solid support. R^5 is H or an alkyl, which is unsubstituted or substituted. R^6 is a protecting group, an amidoalkyl in which the nitrogen atom thereof is 2, 4, or 5 carbon atoms removed from the

20   oxygen of OR^6, an alkyl, an alkyl ketone, an alkenyl, an alkynyl, a cycloalkyl, an aryl, or an aralkyl, wherein R^6 is unsubstituted or substituted. R^{15} is H or a protecting group. Q and Q^1 are the same or different and each is a nucleoside, a oligonucleotide comprising a nucleoside, or

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an oligomer comprising a nucleoside, wherein the
nucleoside is of the formula:

wherein:

B is a labeling group, an alkyl, an alkenyl, an
alkynyl, a cycloalkyl, an aryl, a heteroaryl, a
heterocycloalkyl, an aralkyl, an amino, an alkylamino, a
dialkylamino, a purine, a pyrimidine, adenine, guanine,
cytosine, uracil, or thymine, wherein B is unsubstituted
or substituted, and E is H, a halogen, OR^{13}, NHR^{13}, $NR^{13}R^{14}$,
wherein R^{13} and R^{14} are the same or different and each is
H, a protecting group, an alkyl, or an acyl. X and X^1
are the same or different and each is O, S, or Se, and n
is an integer from 1 to about 300. Q can be the same or
different in each of the units defined by n of formula
(III), when n is greater than 1.

The present invention further provides a method of
preparing a polymer, including the steps of:

(a) reacting a nucleophile that can displace the N-
acyl group of an N-acylphosphoramidite with an N-
acylphosphoramidite of formula (I) or (II), wherein R^4 is
a protecting group, preferably in the presence of a base,
to produce an adduct of the N-acylphosphoramidite and the
nucleophile, which adduct comprises a tricoordinated
phosphorus atom;

(b) reacting the adduct with a reagent selected from
the group consisting of oxidizing agents, sulfurizing
agents, and selenizing agents, to produce a product,

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wherein the tricoordinated phosphorus atom is converted
into a tetracoordinated phosphorus atom; and

(c) removing R^4 from the product to produce another
adduct comprising a nucleophilic substituent. The method
5   can further comprise repeating steps (a) through (c), one
or more times as necessary, until a polymer of specified
length is obtained.

10   BRIEF DESCRIPTION OF THE DRAWINGS
Fig. 1 illustrates the synthesis of an N-
acylphosphoramidite.

Fig. 2 illustrates a solid phase synthesis of an
oligonucleotide.
15   Fig. 3 illustrates the solid phase stereocontrolled
synthesis of phosphorothioate oligonucleotides.

Fig. 4 illustrates the synthesis of various
compounds of formula (I).

Fig. 5 illustrates the preparation of acyclic N-
20   acylphosphoramidites and their application in solid phase
syntheses.

Fig. 6 illustrates an oligonucleotide synthesis
using an alternative method.

Fig. 7 illustrates the preparation of a particular
25   oligonucleotide using either cyclic or acyclic N-
acylphosphoramidites.

Fig. 8A illustrates the structure of a standard
phosphoramidite coupling reagent used in conventional
nucleotide coupling reactions.

30   Fig. 8B illustrates the structure of a P-chiral (S_p)
N-acylphosphoramidite of the present invention.

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Fig. 8C illustrates the structure of a P-chiral (R_P) N-acylphosphoramidite of the present invention.

Fig. 9A illustrates the structure of a standard phosphoramidite coupling reagent used in conventional

5   nucleotide coupling reactions.

Fig. 9B illustrates the structure of a P-diastereomeric (R_P, S_P) N-acylphosphoramidite of the present invention.

Fig. 10A illustrates the HPLC chromatogram for a

10   mixture of the four possible P-diastereomeric oligonucleotide phosphorothioate trimers $d(C_{PS}C_{PS}C)$ prepared using standard phosphoramidite chemistry.

Fig. 10B illustrates the HPLC chromatogram for the pure S_P, S_P diastereomer of $d(C_{PS}C_{PS}C)$ prepared in accordance

15   with the present invention.

Fig. 10C illustrates the HPLC chromatogram obtained by co-injecting the pure S_P, S_P diastereomer of $d(C_{PS}C_{PS}C)$ with the mixture containing all four possible P-diastereomers.

20   Fig. 10D illustrates the HPLC chromatogram for the pure R_P, R_P diastereomer of $d(C_{PS}C_{PS}C)$, prepared in accordance with the present invention.

Fig. 10E illustrates the HPLC chromatogram obtained by co-injecting the pure R_P, R_P diastereomer of $d(C_{PS}C_{PS}C)$

25   with the mixture containing all four possible P-diastereomers.

Fig. 11A illustrates the HPLC chromatogram for a mixture of the eight possible P-diastereomeric oligonucleotide phosphorothioate tetramers of $d(C_{PS}C_{PS}C_{PS}C)$

30   prepared using standard phosphoramidite chemistry.

Fig. 11B illustrates the HPLC chromatogram for the pure R_P, S_P, R_P diastereomer of $d(C_{PS}C_{PS}C_{PS}C)$ prepared in accordance with the present invention.

Fig. 11C illustrates the HPLC chromatogram obtained by co-injecting the pure R_P, S_P, R_P diastereomer of $d(C_{PS}C_{PS}C_{PS}C)$ with the mixture containing all eight possible P-diastereomers.

Fig. 12A illustrates the HPLC chromatogram for the dimeric phosphodiester $d(T_{PO}G)$ prepared under standard phosphoramidite coupling conditions in the absence of moisture.

Fig. 12B illustrates the HPLC chromatogram for the product obtained in the preparation of dimeric phosphodiester $d(T_{PO}G)$ under standard phosphoramidite coupling conditions in the presence of moisture (0.1% water).

Fig. 12C illustrates the HPLC chromatogram for the dimeric phosphodiester $d(T_{PO}G)$ prepared by an N-acylphosphoramidite coupling reagent of the present invention in the absence of moisture.

Fig. 12D illustrates the HPLC chromatogram for the product obtained in the preparation of dimeric phosphodiester $d(T_{PO}G)$ by an N-acylphosphoramidite coupling reagent of the present invention in the presence of moisture (0.1% water).

Fig. 13A illustrates a general example of a thermal cleavage of the bond linking an organic moiety to a non-bridging phosphate or phosphorothioate oxygen.

Fig. 13B illustrates specific examples thermal cleavage of the bond linking an organic moiety to a non-bridging phosphate or phosphorothioate oxygen.

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DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is predicated, at least in part, on the surprising and unexpected discovery that the utilization of N-acylphosphoramidites as a coupling vehicle, for example, with respect to the coupling of nucleoside-containing fragments, occurs without any epimerization at phosphorus. Moreover, post-coupling reactions and transformations (i.e., the synthetic steps that are carried out after step (b) of the method as set forth below), for example, oxidation, sulfurization, and deprotection, occur without epimerization at the phosphorus atom.

The present inventive method of synthesizing polymers has tremendous synthetic advantages that are unprecedented in the art, particularly with respect to the synthesis of oligonucleotides, in that it enables the facile production of P-chiral oligomeric or polymeric products, with complete control of stereochemistry with respect to the phosphorus atom. Moreover, stereochemistry can be controlled for tricoordinated and tetracoordinated phosphorus atoms.

Although applicants do not wish to be bound by any one particular theory, it is believed that the N-acyl functionality of the N-acylphosphoramidite ring (which functions as a leaving group in coupling step (b) of the method as set forth below) is not labile under the coupling conditions utilized for the displacement thereof; rather, displacement occurs via a purely bimolecular nucleophilic mechanism. As such, there is no

"scrambling" or epimerization of the phosphorus atom in
the coupling step.

By contrast, the standard phosphoramidite approach
presently utilized in the art involves displacement of an
5   amino functionality on phosphorus, and requires acidic
conditions for the displacement thereof. The phosphorus-
nitrogen bond in a standard phosphoramidite is labile
under acidic conditions (even when a mild acid such as
tetrazole is used), invariably resulting in epimerization
10   of the phosphorus atom in the resulting coupled adduct.
Although attempts have been made to control the extent of
epimerization in coupling reactions using
phosphoramidites, there is inevitably some epimerization,
which promotes the formation of diastereomers. Even if
15   the formation of undesired diastereomers occurs in minute
quantities, the overall yield of the target product
decreases exponentially.

The compounds and methods of the present invention,
therefore, provide for the stereospecific substitution of
20   tricoordinated phosphorus compounds under basic
conditions. In this regard, the monomeric compounds of
the present invention (preferably of formulae (I) and
(II)), and the oligomeric compounds of the present
invention (preferably of formula (III)), are particularly
25   useful in the synthesis of polymers, particularly
oligonucleotide polymers.

Generally, the compounds of the present invention
are hydroxyl-protected monomer-*O*-(*O*-protected)-(*N*-
acyl)phosphoramidites, or hydroxyl protected
30   oligomer/polymer-*O*-(*O*-protected)-(*N*-
acyl)phosphoramidites, exemplified by formulae (I)-(III).

In a preferred embodiment, the compound is a hydroxyl-protected monomer-O-(N-acyl)-1,3,2-substituted oxazaphospholane (formula (I)), which can be isolated as the Rp or Sp chiral form, to be used in the synthesis of

5   polymers containing stereogenic phosphorus centers of predetermined configuration in a site-specific manner.

In view of the above, the present invention provides a compound of the formula:

10   (I)   (II),   or

(III).
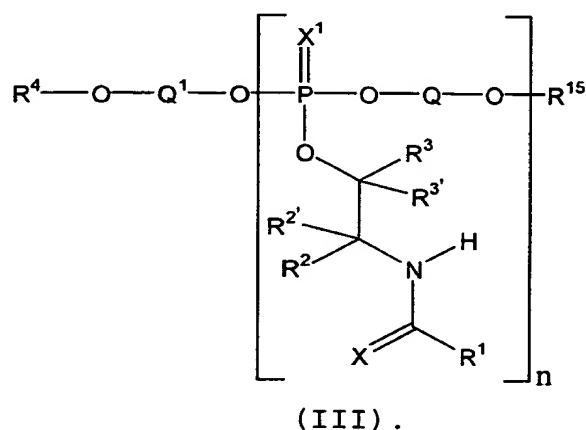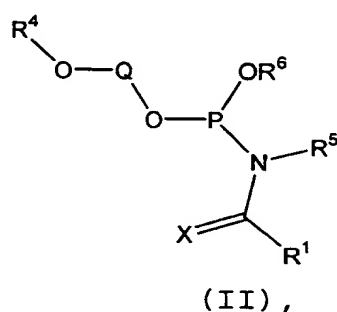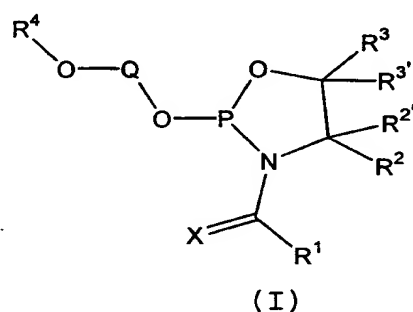
With respect to the above formulae, any suitable

15   acyl moiety can be used. Suitable acyl moieties include R^1(C=X)N- groups which render the phosphorus-(N-acyl) bond sufficiently reactive to allow displacement of the N-acyl group by a nucleophile, preferably under basic conditions. Preferably, R^1 is an alkyl (e.g., a C_1-C_6

20   alkyl), an alkenyl (e.g., a C_2-C_6 alkenyl), an alkynyl (e.g., a C_2-C_6 alkynyl), a cycloalkyl (e.g., a C_3-C_7

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cycloalkyl), an aryl (e.g., phenyl or naphthyl), or an
aralkyl (e.g., benzyl, phenethyl, phenylpropyl, or the
like), wherein R^1 is unsubstituted or is substituted with
one or more substituents, which are the same or

5   different, selected from the group consisting of R^7, OR^7,
SR^7, NR^8COR^7, NR^8CSR^7, $NR^8CO_2R^7$, $NR^8C(O)SR^7$, $NR^8CS_2R^7$, O_2CR^7,
S_2CR^7, $SCOR^7$, $OCSR^7$, SO_2R^7, OSO_2R^7, $NR^8SO_2R^7$, CN, NO_2, N_3, and
a halogen, wherein R^7 is an alkyl, an aryl, or an
aralkyl, wherein R^7 is unsubstituted or is substituted

10   with one or more halogen atoms, which are the same or
different, and R^8 is H or an alkyl. In one embodiment, R^1
is an alkyl, which is preferably a C_1-C_6 alkyl, that is
unsubstituted or is substituted with one or more
substituents, which are the same or different, selected

15   from the group consisting of fluorine, OR^7 and SR^7,
wherein R^7 is an alkyl or an aryl. When R^1 is a C_1-C_6
alkyl, it is more preferably a C_1-C_3 alkyl which is
unsubstituted or substituted with one or more fluorine
atoms, for example, a methyl, optionally substituted with

20   one or more fluorine atoms, most preferably fluoromethyl.
When R^1 is a C_1-C_6 alkyl substituted with OR^7 or SR^7, R^1 is
most preferably a methoxymethyl, a methylthiomethyl or a
phenoxy.

     While R^2 and $R^{2'}$ can be any suitable substituent,

25   preferably R^2 and $R^{2'}$ are the same or different and each
is H, an alkyl, an alkenyl, an alkynyl, a cycloalkyl, an
aryl or an aralkyl. When R^2 or $R^{2'}$ is an alkyl, it is
preferably a C_1-C_6 alkyl. When R^2 or $R^{2'}$ is an alkenyl, it
is preferably a C_2-C_6 alkenyl. When R^2 or $R^{2'}$ is an

30   alkynyl, it is preferably a C_2-C_6 alkynyl. When R^2 or $R^{2'}$
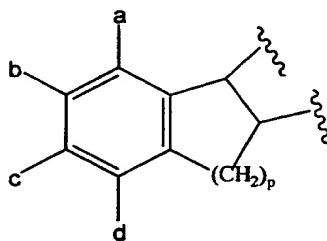is a cycloalkyl, it is preferably a C_3-C_7 cycloalkyl. R^2

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and/or $R^{2'}$ can be unsubstituted or substituted with one or more substituents selected from the group consisting of OR^7, CN, NO_2, N_3, and a halogen. In a particularly preferred embodiment, R^2 and $R^{2'}$ are hydrogen.

5   While R^3 and $R^{3'}$ can be any suitable substituent, preferably R^3 and $R^{3'}$ are the same or different and each is H, an alkyl, an alkenyl, an alkynyl, a cycloalkyl, an aryl or an aralkyl. When R^3 or $R^{3'}$ is an alkyl, it is preferably a C_1-C_6 alkyl. When R^3 or $R^{3'}$ is an alkenyl, it

10   is preferably a C_2-C_6 alkenyl. When R^3 or $R^{3'}$ is an alkynyl, it is preferably a C_2-C_6 alkynyl. When R^3 or $R^{3'}$ is a cycloalkyl, it is preferably a C_3-C_7 cycloalkyl. R^3 and/or $R^{3'}$ can be unsubstituted or substituted with one or more substituents selected from the group consisting of a

15   trialkylsilyl, an aryldialkylsilyl, an alkyldiarylsilyl, CN, NO_2, N_3, a halogen, OR^7, $P(O)(OR^7)(OR^8)$, COR^9, CSR^9, CO_2R^9, $COSR^9$, $CSOR^9$, $CONR^8R^9$, $CSNR^8R^9$, SO_2R^9, and $SO_2NR^8R^9$, wherein R^7 is as defined herein. While R^9 can be any suitable substituent, R^9 preferably is H, an alkyl, an

20   alkenyl, an alkynyl, a cycloalkyl, an aryl or an aralkyl. When R^9 is an alkyl, it is preferably a C_1-C_6 alkyl. When R^9 is an alkenyl, it is preferably a C_2-C_6 alkenyl. When R^9 is an alkynyl, it is preferably a C_2-C_6 alkynyl. When R^9 is a cycloalkyl, it is preferably a C_3-C_7 cycloalkyl.

25   R^9 can be unsubstituted or it can be substituted with one or more substituents selected from the group consisting of CN, NO_2, N_3, and a halogen.

There are certain preferred combinations of $R^1-R^{3'}$. In one preferred embodiment, R^3 is a phenyl, R^2, $R^{2'}$ and $R^{3'}$

30   all are H, and R^1 is selected from the group consisting of methyl, fluoromethyl, methoxymethyl and phenoxymethyl.

In another preferred embodiment, R^3 is a vinyl, R^2 is H, and R^1 is selected from the group consisting of methyl, fluoromethyl, methoxymethyl and phenoxymethyl. In yet another preferred embodiment, R^3 and R^2 are both H, and R^1

5   is a methyl.

   Alternatively, R^2 and R^3, R^2 and $R^{3'}$, $R^{2'}$ and R^3, and $R^{2'}$ and $R^{3'}$, together with the carbon atoms to which they are bonded, can comprise a cyclic substituent. In other words, a combination of either of R^2 and $R^{2'}$ with either

10 of R^3 and $R^{3'}$ can comprise a cyclic substituent. Preferably, R^2 or $R^{2'}$ and R^3 or $R^{3'}$, together with the carbon atoms to which they are bonded, comprise a cyclic substituent of the formula:

15 wherein p is an integer from 0-6 and a-d are the same or different and each is selected from the group consisting of H, an alkyl, a nitro, an amino, a hydroxy, a thio, a cyano and a halogen. In a preferred embodiment, p is 1 and all of a-d are H. In a particularly preferred

20 embodiment, p is 1, all of a-d are H, and R^1 is fluoromethyl.

   R^4 is a protecting group or a solid support.

   R^5 is H or an alkyl, preferably a C_1-C_3 alkyl, which can be unsubstituted or substituted with one or more

25 substituents, which are the same or different, selected from the group consisting of OR^7, CN, NO_2, N_3, and a halogen, wherein R^7 is as defined herein.

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While R^6 can be any suitable substituent, R^6 preferably is a protecting group, an alkyl, an amidoalkyl, an alkenyl, an alkynyl, a cycloalkyl, an aryl, an alkyl ketone, or an aralkyl. When R^6 is an

5   alkyl, it is preferably a C_1-C_6 alkyl. When R^6 is an amidoalkyl, it is preferably an amidoalkyl, more preferably a C_1-C_6 amidoalkyl, in which the nitrogen atom thereof is 2, 4, or 5 atoms removed from the oxygen atom of OR^6, and is most preferably an amidoalkyl whose amide

10   is easily hydrolyzed or cleaved and liberates an amine which is separated from the oxygen of OR^6 by 2, 4, or 5 carbon atoms. When R^6 is an alkenyl, it is preferably a C_2-C_6 alkenyl. When R^6 is an alkynyl, it is preferably a C_2-C_6 alkynyl. When R^6 is a cycloalkyl, it is preferably

15   a C_3-C_7 cycloalkyl. R^6 can be unsubstituted or substituted with one or more substituents, which are the same or different, selected from the group consisting of CN, NO_2, N_3, and a halogen.

R^{15} is H or a protecting group.

20   Q and Q^1 are the same or different and each is a nucleoside, an oligonucleotide comprising a nucleoside, or an oligomer comprising a nucleoside (e.g., an oligonucleotide or the like). Q and/or Q^1 can be a natural nucleoside or a modified/unnatural nucleoside. Q

25   and/or Q^1 also can be an oligomer comprising one or more natural or modified/unnatural nucleosides. Modified nucleosides can be obtained, for example, by any suitable synthetic method known in the art for preparing nucleosides, derivatives, or analogs thereof. Modified

30   nucleosides include, but are not limited to, chemically modified nucleosides used as building blocks for

"labeled" oligonucleotides, or suitable precursors or analogs used in the preparation of such modified nucleosides. Various chemically modified nucleosides are described, for example, in Smith et al., *Nucleosides &*

5   *Nucleotides*, *15*(10), 1581-1594 (1996) ("Smith et al."). Smith et al. describes the synthesis of nucleosides (and oligomers which include such nucleosides) in which the base ring is replaced by a carboxylic acid to which is appended various "labeling" groups (e.g., biotin,

10   cholesterol, fluorenylmethoxycarbonyl (Fmoc), and trifluoroacetyl) via a modified amide linker. Modified nucleosides also include other chemically modified nucleosides, for example, nucleosides described in Smith et al. in which the base ring is replaced by a

15   hydroxyethyl, a cyano, or a carboxylic acid (including esters and amides thereof). Modified nucleosides further include nucleosides in which the base ring is replaced by a cyclic substituent, for example, an aryl, a cycloalkyl, a heterocycloalkyl, or a heteroaryl (other than a base

20   naturally occurring in nucleosides).

        Q and/or Q^1 also include oligonucleotides, which can be natural or modified. Modified oligonucleotides include, for example, oligonucleotides containing a modified nucleoside (as described herein),

25   oligonucleotides containing a modified internucleotide linkage, or oligonucleotides having any combination of modified nucleosides and internucleotide linkages (even if a natural nucleoside is present in the oligomer chain). Oligonucleotides whose nucleosides are connected

30   via modified internucleotide linkages can be found, for example, in Waldner et al., *Bioorg. Med. Chem. Letters*,

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6, 19, 2363-2366 (1996) ("Waldner et al."), which
describes the synthesis of oligonucleotides containing
various amide internucleotide linkages.

In a preferred embodiment, Q and Q^1 are the same or
5   different and each is a nucleoside substituent (or an
oligonucleotide comprising a nucleoside, a nucleoside, or
an oligomer comprising a nucleoside) of the formula:

or
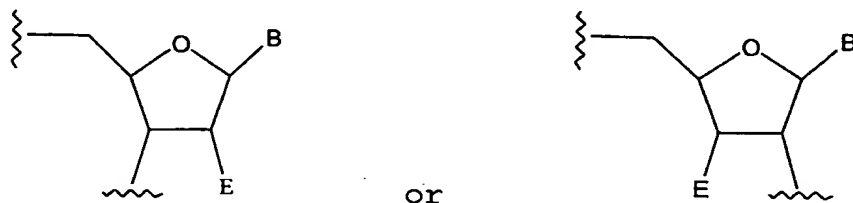
wherein B is a labeling group, an alkyl, an alkenyl, an
10   alkynyl, a cycloalkyl, an aryl, a heteroaryl, a
heterocycloalkyl, an aralkyl, an amino, an alkylamino, a
dialkylamino, a purine, a pyrimidine, adenine, guanine,
cytosine, uracil, or thymine, wherein B is unsubstituted
or substituted with one or more substituents, which are
15   the same or different, selected from the group consisting
of a protecting group, R^{11}, OR^{11}, NHR^{11}, $NR^{11}R^{12}$, CN, NO_2, N_3,
and a halogen, wherein R^{11} and R^{12} are the same or
different and each is H, a protecting group, or an alkyl;
and E is H, a halogen, OR^{13}, NHR^{13}, or $NR^{13}R^{14}$, wherein R^{13}
20   and R^{14} are the same or different and each is H, a
protecting group, an alkyl, or an acyl. When B is an
alkyl, preferably it is a C_1-C_6 alkyl. When B is an
alkenyl, it is preferably a C_2-C_6 alkenyl. When B is an
alkynyl, preferably it is a C_2-C_6 alkynyl. When B is a
25   cycloalkyl, preferably it is a C_3-C_7 cycloalkyl. When R^{13}
and/or R^{14} is an alkyl, preferably it is a C_1-C_6 alkyl.

As indicated above, the Q in the N-
acylphosphoramidites of formulae (I) and (II), and the Q

and Q^1 in the intermediates obtained therefrom (formula (III)), include nucleosides (natural and modified) and oligomers which include one or more of such nucleosides. Any suitable monomer-monomer, monomer-oligomer, oligomer-

5   monomer, or oligomer-oligomer coupling reaction can be accomplished, stereospecifically, using the compounds and methods of the present invention. For example, the N-acylphosphoramidite of formula (I) or (II) can be used to stereospecifically couple a suitably protected nucleoside

10   (or even a suitably protected oligonucleotide) to an oligonucleotide. Thus, the N-acylphosphoramidite of the present invention can be attached to an oligomer such as, for example, an oligonucleotide (i.e., wherein Q is an oligonucleotide), as well as a monomer (i.e., wherein Q

15   is a nucleoside). The nucleophile which is coupled to an N-acylphosphoramidite of the present invention also can be monomeric or oligomeric. Accordingly, Q^1 also includes oligomers that contain, as a component thereof, a nucleoside substituent as described herein.

20   The C=X bond of the N-acylphosphoramidites of the present invention includes carbonyl and carbonyl equivalents. Thus, the N-acyl group includes carbonyl (wherein X is O), thiocarbonyl (wherein X is S), and selenocarbonyl (wherein X is Se). Typically, the N-acyl

25   group is a carbonyl, wherein X is O.

        Examples of monomeric compounds of the present invention include compounds of the formulae:

20
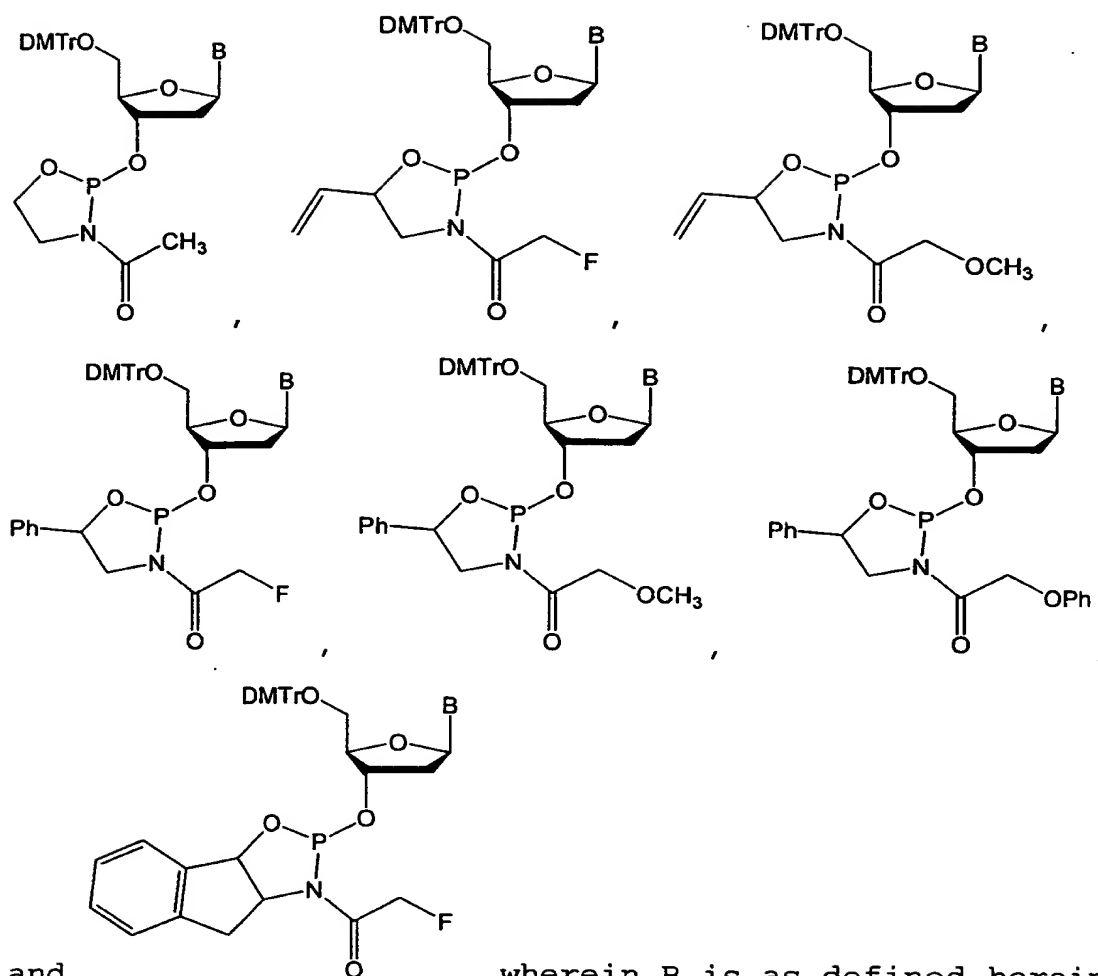
and , wherein B is as defined herein.

Stereospecific coupling reactions can be carried out

5   successively "n" times, for example, starting with a

nucleophile R^4-O-Q^1-OH (wherein R^4 and Q^1 are as defined

herein), and continuing thereafter, to provide an

intermediate of formula (III), wherein n is an integer

from 1 to about 300. It will be appreciated that when a

10   compound of formula (I) is reacted with a nucleophile

R^4-O-Q^1-OH, then "R^4" of formula (I) is represented by

"R^{15}" of formula (III). When the protecting group R^{15} is

removed, then R^{15} becomes a hydrogen. R^4 and R^{15} desirably

are not both solid supports in formula (III). When R^{15} is

15   hydrogen, then another coupling reaction can be carried

out, and the process repeated successively, until a

polymer of desired length or structure is obtained. In
each successive reaction, the Q substituent of formula
(I) can be the same or different, as desired, to obtain a
variety of different combinations. As such, Q can be the
same or different in each of the units defined by n, when
n is greater than 1. Preferably, n is in the range of
from about 3 to about 200; more preferably, n is in the
range from about 10 to about 40; and most preferably n is
in the range from about 15 to about 25.

In a preferred embodiment, Q and/or Q^1 is a
nucleoside substituent of the formula:

or
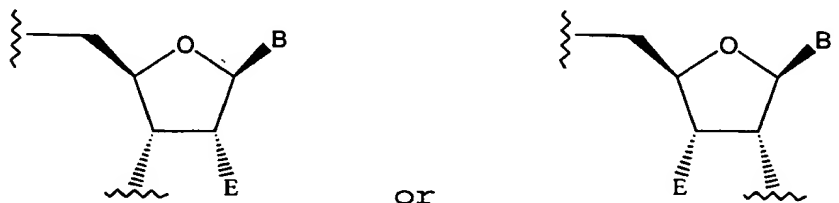
In this embodiment, R^4 is advantageously a solid
support or a protecting group. The protecting group is
most preferably a 4,4'-dimethoxytrityl protecting group.

As utilized herein, the term "alkyl" means a
straight-chain or branched-chain alkyl radical which,
unless otherwise specified, contains from about 1 to about
20 carbon atoms, preferably from about 1 to about 10
carbon atoms, more preferably from about 1 to about 8
carbon atoms, and most preferably from about 1 to about 6
carbon atoms. Examples of such alkyl radicals include
methyl, ethyl, propyl, isopropyl, n-butyl, sec-butyl,
isobutyl, tert-butyl, pentyl, isoamyl, hexyl, octyl,
dodecanyl, and the like.

The term "alkenyl" means a straight-chain or
branched-chain alkenyl radical, which has one or more
double bonds and, unless otherwise specified, contains

from about 2 to about 20 carbon atoms, preferably from about 2 to about 10 carbon atoms, more preferably from about 2 to about 8 carbon atoms, and most preferably from about 2 to about 6 carbon atoms. Examples of alkenyl

5   radicals include vinyl, allyl, 1,4-butadienyl, isopropenyl, and the like.

The term "alkynyl" means a straight-chain or branched-chain alkynyl radical, which has one or more triple bonds and contains from about 2 to about 20 carbon

10   atoms, preferably from about 2 to about 10 carbon atoms, more preferably from about 2 to about 8 carbon atoms, and most preferably from about 2 to about 6 carbon atoms. Examples of alkynyl radicals include ethynyl, propynyl (propargyl), butynyl, and the like.

15   The terms "alkylamino" and "dialkylamino" mean an alkyl or a dialkyl amine radical, wherein the term "alkyl" is defined as above. Examples of alkylamino radicals include methylamino ($NHCH_3$), ethylamino ($NHCH_2CH_3$), n-propylamino, isopropylamino, n-butylamino, isobutylamino,

20   sec-butylamino, $tert$-butylamino, n-hexylamino, and the like. Examples of dialkylamino radicals include dimethylamino ($N(CH_3)_2$), diethylamino ($N(CH_2CH_3)_2$), di-n-propylamino, diisopropylamino, di-n-butylamino, diisobutylamino, di-sec-butylamino, di-$tert$-butylamino,

25   di-n-hexylamino, and the like.

The term "cycloalkyl" means a monocyclic alkyl radical, or a polycyclic alkyl which comprises one or more alkyl carbocyclic rings, which can be the same or different when the polycyclic radical has 3 to about 10

30   carbon atoms in the carbocyclic skeleton of each ring. Preferably, the cycloalkyl has from about 4 to about 7

23

carbon atoms, more preferably from about 5 to about 6 carbons atoms. Examples of monocyclic cycloalkyl radicals include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, cyclodecyl, and the like. Examples of

5   polycyclic cycloalkyl radicals include decahydronaphthyl, bicyclo[5.4.0]undecyl, adamantyl, and the like.

The term "aryl" refers to an aromatic carbocyclic radical, as commonly understood in the art, and includes monocyclic and polycyclic aromatics such as, for example,

10   phenyl and naphthyl radicals, which radicals are, unless indicated otherwise, unsubstituted or substituted with one or more substituents selected from the group consisting of a halogen, an alkyl, an alkoxy, an amino, a cyano, a nitro, and the like. Preferably, the aryl has one or more

15   six-membered carbocyclic rings including, for example, phenyl, naphthyl, and biphenyl, and are unsubstituted or substituted as set forth herein.

The term "aralkyl" means alkyl as defined herein, wherein an alkyl hydrogen atom is replaced by an aryl as

20   defined herein. Examples of aralkyl radicals include benzyl, phenethyl, 1-phenylpropyl, 2-phenylpropyl, 3-phenylpropyl, 1-naphthylpropyl, 2-naphthylpropyl, 3-naphthylpropyl, 3-naphthylbutyl, and the like.

The terms heterocycle and heterocyclic refer to both

25   heterocycloalkyls and heteroaryls. The term "heterocycloalkyl" means a cycloalkyl radical as defined herein (including polycyclics), wherein at least one carbon of a carbocyclic ring is substituted with a heteroatom such as, for example, O, N, or S. The

30   heterocycloalkyl optionally has one or more double bonds within a ring, and may be aromatic, but is not necessarily

aromatic. The heterocycloalkyl preferably has 3 to about
10 atoms (members) in the carbocyclic skeleton of each
ring, preferably from about 4 to about 7 atoms, more
preferably from about 5 to about 6 atoms. Examples of
heterocycloalkyl radicals include epoxy, aziridyl,
oxetanyl, tetrahydrofuranyl, ribose, dihydrofuranyl,
piperidinyl, piperazinyl, pyranyl, morpholinyl, and the
like.

The term "heteroaryl" means a radical defined by an
aromatic heterocyclic ring as commonly understood in the
art, including monocyclic radicals such as, for example,
imidazole, thiazole, pyrazole, pyrrole, furane,
pyrazoline, thiophene, oxazole, isoxazole, pyridine,
pyridone, pyrimidine, cytosine, 5-methylcytosine,
thymine, pyrazine, and triazine radicals, and polycyclics
such as, for example, quinoline, isoquinoline, indole,
purine, adenine, guanine, N^6-methyladenine, and
benzothiazole radicals, which heteroaryl radicals are
unsubstituted or substituted with one or more
substituents, which are the same or different, selected
from the group consisting of a halogen, an alkyl, an
alkoxy, an amino, a cyano, a nitro, and the like. It will
be appreciated that the heterocycloalkyl and the
heteroaryl substituents can be coupled to the compounds of
the present invention via a heteroatom, such as nitrogen
(e.g., 1-imidazolyl). It will also be appreciated that
heteroaryls, as defined herein, are not necessarily
"aromatic" in the same context as phenyl is aromatic,
although heteroaryls nonetheless demonstrate physical and
chemical properties associated with aromaticity, as the
term is understood in the art.

The term "nucleoside" includes all modified and naturally occurring nucleosides, including all forms of furanosides found in nucleic acids. Naturally occurring nucleosides include, for example, adenosine, guanosine,

5   cytidine, thymidine, and uridine.

Nucleoside "derivatives" or "analogs" include synthetic nucleosides as described herein. Nucleoside derivatives also include nucleosides having modified base moieties, with or without protecting groups. Such

10   analogs include, for example, deoxyinosine, 2,6-diaminopurine-2'-deoxyriboside, 5-methyl-2'-deoxycytidine, and the like. The base rings most commonly found in naturally occurring nucleosides are purine and pyrimidine rings. Naturally occurring purine

15   rings include, for example, adenine, guanine, and N^6-methyladenine. Naturally occurring purine rings include, for example, cytosine, thymine, and 5-methylcytosine. The compounds and methods of the present invention include such base rings and synthetic analogs thereof, as

20   well as unnatural heterocycle-substituted base sugars, and even acyclic substituted base sugars. Moreover, nucleoside derivatives include other purine and pyrimidine derivatives, for example, halogen-substituted purines (e.g., 6-fluoropurine), halogen-substituted

25   pyrimidines, N^6-ethyladenine, N^6-(alkyl)-cytosines, 5-ethylcytosine, and the like.

The term "oligonucleotide" as used herein includes linear oligomers of natural or modified nucleosides, and modified ologonucleotides, as described herein.

30   Oligonucleotides include deoxyribonucleosides, ribonucleosides and anomeric forms thereof, and the like.

26

Oligonucleotides are typically linked by phoshodiester

bonds, or the equivalent thereof, ranging in size from a

few monomeric units (e.g., 3 or 4) to several hundred

monomeric units. Preferably, the oligonucleotides of the

5   present invention are oligomers of naturally-occurring

nucleosides ranging in length from about 12 to about 60

monomeric units, and more preferably, from about 15 to

about 30 monomeric units. Whenever an oligonucleotide is

represented by a sequence of letters, such as "AGTC" it

10   will be appreciated that the nucleotides are in the 5'-3'

orientation from left to right.

Phosphorus linkages between nucleosidic monomers

include phosphodiester bonds and analogs of

phosphodiester bonds, such as phoshorothioate,

15   phosphoroselenoate, alkylphosphonate, and

phosphoramidate. Preferably, the monomers of the

oligonucleotides of the present invention are linked by

phosphodiester, phosphorothioate, methanephosphonate, or

phosphoramidate linkages.

20   The term "oligomer comprising a nucleoside" as

utilized herein means an oligomer in which at least one

of the monomeric units comprises nucleoside, and at least

one of the other monomeric units is not a nucleoside.

For example, one of the monomeric units in the oligomer

25   can be an amino acid, an organic spacer (e.g., an

aliphatic or aromatic spacer, an alkylene glycol, or the

like), or a carbohydrate (e.g., a sugar). Moreover, one

of the non-nucleoside units of the oligomer can itself be

oligomeric, for example, a peptide, an oligosaccharide, a

30   polyalkylene glycol, or the like.

Any suitable protecting group (sometimes referred to as a blocking group) can be utilized in accordance with the present invention. The term "protecting group" as used herein means a substituent, a functional group, a
5   salt, a ligand, or the like, which is bonded (e.g., via covalent bond, ionic bond, or complex) to a potentially reactive functional group and prevents the potentially reactive functional group from reacting under certain reaction conditions. Potentially reactive functional
10   groups include, for example, amines, carboxylic acids, alcohols, double bonds, and the like. Preferably, the protecting group is stable under the reaction conditions for which the protecting group is employed, and also can be removed under reasonably mild deprotection conditions.
15   It will be appreciated that the protecting group to be used in accordance with the present invention depends on the type of substituent that is being protected. Thus, it is not uncommon to use a different protecting group for each of a phosphite oxygen, a phosphate oxygen, an
20   amine, a thiol, a hydroxyl, and the like. It will also be appreciated that the choice of protecting groups will depend on other factors such as, for example, the reaction conditions employed in a particular synthetic step, the pH, the temperature, and the relative
25   reactivities of the reactants and/or products.

Protecting groups for hydroxyls include, for example, silyl ethers (e.g., trimethylsilyl, triethylsilyl, *tert*-butyldimethylsilyl, dimethylphenylsilyl, and diphenylmethylsilyl), benzyl
30   carbonates, trityl, monomethoxytrityl, dimethoxytrityl, esters (e.g., acetate, benzoate, and the like), pixyl,

28

tert-butyloxycarbonyl, 9-fluorenylmethyloxycarbonyl
(Fmoc), a tetrahydropyranyl group, and the like. When
the hydroxyl is a sugar hydroxyl, preferred protecting
groups include, for example, pixyl, acetyl, 9-

5   fluorenylmethyloxycarbonyl (Fmoc), t-butyldimethylsilyl
(TBDMS), trityl, monomethoxytrityl ("MMT" or "MMTr"),
dimethoxytrityl ("DMT" or "DMTr"), and the like.

Protecting groups for nitrogen include, for example,
amides (e.g., trifluoroacetyl, benzoyl, and isobutyryl),

10 carbamates (e.g., *tert*-butyloxycarbonyl and N-
benzyloxycarbonyl), trityl, and the like. When an amine
to be protected is part of a nucleoside base ring,
suitable protecting groups can include amides, for
example, benzoyl, isobutyryl, and the like.

15   The term "carboxyl" means any functional group with
a carbonyl backbone, and includes functional groups such
as, for example, a carboxylic acid, an esters (e.g.,
ethoxycarbonyl), and amides (e.g., benzamido).

Any suitable solid support can be used in the

20 compounds and methods of the present invention. Solid
supports are commonly known in the art and include, for
example, organic solid supports (e.g., crosslinked
polystyrene) and inorganic solid supports. Preferably,
the solid support is inorganic, and is more preferably a

25 silica support. It will be appreciated that the solid
support includes all linkers, spacers, arms, and other
moieties (organic or inorganic) known in the art for
manipulating attachment to a solid support. It will also
be appreciated that the solid support can be bonded to

30 the molecule directly, without using any of the aforesaid
linkers, spacers, arms, or other connecting moieties.

29

Some aspects of the invention are common with known
approaches to solid phase synthesis of oligonucleotides,
for example, selection of suitable protecting groups,
selection of suitable solid phase supports, and the like.

5　Consequently, considerable guidance in making such
selections in the context of the present invention can be
found in literature, e.g. Beaucage et al., *Tetrahedron*,
49, 6123-6194 (1993).

Typically, the monomeric units in the polymers
10　prepared in accordance with the present invention are
connected via phosphorus diester linkages, for example,
phosphate or chiral phosphate (P-chiral) linkages, as
desired. However, the compounds and methods of the
present invention are not limited to the synthesis of
15　polymers having only phosphorus-linked monomeric units.
For example, the compounds of the present invention also
can be used to introduce one or more phosphorus-linked
units into a polymer having another type of linkage in
the structure thereof, for example, a carbonate, a urea,
20　an ester, an ether, or any suitable combination thereof.

In a preferred embodiment, the compound of the
present invention is a cyclic N-acylphosphoramidite of
the formula:

, or

,
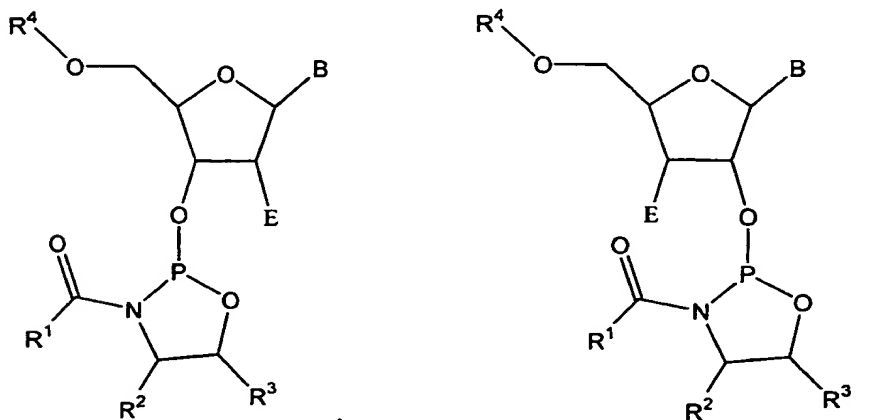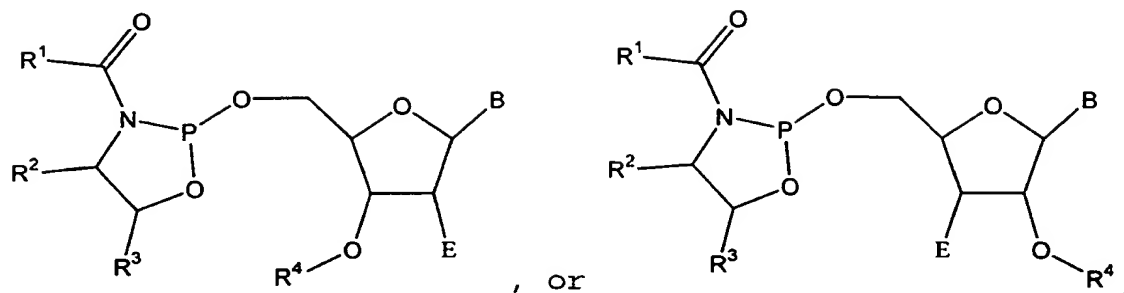
wherein R^1-R^4, B, and E are as defined herein.

Particular substituents for R^1, R^2, $R^{2'}$, R^3 and/or $R^{3'}$

5   (formulae (I) or (III)), or R^6 (formula (II)) can be
selected which have a structure that facilitates removal
of the organic moiety remaining on the non-bridging
phosphate or phosphorothioate oxygen after coupling has
been carried out in accordance with the present

10   invention. For example, R^1 advantageously can be an
alkyl group substituted with a suitably positioned
nucleophile that is protected with an easily removable
protecting group (e.g., a "latent" internal nucleophile).
When the nucleophile is released upon deprotection, the

15   nucleophile on R^1 is positioned such that it can
intramolecularly attack (and therefore cleave) the bond
linking the organic moiety to the non-bridging phosphate,
phosphorothioate or phosphoroselenoate oxygen. For
example, when R^1 is trifluoroacetamido, the

20   trifluoroacetyl group can be easily removed, thereby
liberating an amine (nucleophile) which, in turn,
intramolecularly cleaves the organic moiety from the non-
bridging phosphate, phosphorothioate or
phosphoroselenoate oxygen as illustrated below in Scheme

25   1.
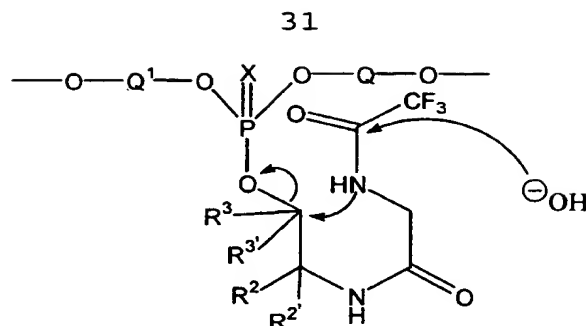
Scheme 1

Other suitably protected "latent" nucleophiles (in
addition to an amine, as illustrated in Scheme 1) can be
5   used in this fashion with respect to R^1, for example, a
protected hydroxyl (e.g., an ester or a silyl ether) or a
protected thiol (e.g., acetylthio).

Similarly, R^1, R^2, $R^{2'}$, R^3, and/or $R^{3'}$ (formulae (I) or
(III)), or R^6 (in formula (II)) can be advantageously
10   chosen to promote cleavage of the bond linking the
organic moiety to the non-bridging phosphate,
phosphorothioate or phosphoroselenoate oxygen by other
mechanisms. The properties of R^1, R^2, $R^{2'}$, R^3, $R^{3'}$, R^5
and/or R^6 can promote cleavage of the bond linking the
15   organic moiety to the non-bridging phosphate,
phosphorothioate or phosphoroselenoate oxygen. For
example, it is believed that if R^2 and/or $R^{2'}$ is an
electron-withdrawing group, an olefin, or an aryl (e.g.,
phenyl) substituent, such groups will promote cleavage of
20   the bond linking the organic moiety to the non-bridging
phosphate, phosphorothioate or phosphoroselenoate oxygen
via elimination (i.e., because these groups promote
elimination and formation of a double bond between the
carbons bearing R^2 and R^3). In the same manner, R^3, $R^{3'}$
25   and/or R^6 advantageously can be a group that promotes
cleavage of the bond linking the organic moiety to the
non-bridging phosphate, phosphorothioate or

phosphoroselenoate oxygen. For example, R^3 and/or $R^{3'}$ and/or $R^{3''}$ can be an electron-withdrawing group, an olefin, an aryl substituent (e.g., phenyl), or an aralkyl (e.g., benzyl), to promote cleavage via elimination.

5   Alternatively, when an acyclic N-acylphosphoramidite is used (formula (II)), R^6 can be a group that promotes cleavage via displacement of an external nucleophile (e.g., when R^6 is methyl) or via elimination (e.g., when R^6 is cyanoethyl, 4-nitrophenyl, nitroethyl, or the

10   like). Alternatively, R^6 can be a group that promotes cleavage via intramolecular nucleophilic displacement (by the mechanism illustrated in Scheme 1), for example, when R^6 is an amidoalkyl whose amide can be hydrolyzed to liberate an amine that is separated from the oxygen of

15   OR^6 by 2, 4, or 5 carbon atoms. Such amidoalkyl R^6 groups can include, for example, $(CH_2)_mN(R^8)COCH_2F$, $(CH_2)_mN(R^8)COCHF_2$, and $(CH_2)_mN(R^8)COCF_3$, wherein m is 2, 4, or 5, and R^8 is H or C_1-C_6 alkyl. Alternatively, R^2, R^3, $R^{2'}$, $R^{3'}$, R^5 and/or R^6 can be substituted with a silyl group

20   that promotes elimination when the silyl group is removed. For example, R^6 can be a 2-(tri-organosilyl)-ethyl group, or R^3 and/or $R^{3'}$ can be a tri-organosilylmethyl group. Preferred silyl groups include, for example, trialkylsilyl (e.g., trimethylsilyl and

25   triethylsilyl), aryldialkylsilyl (e.g., dimethylphenylsilyl), and alkyldiarylsilyl (e.g., methyldiphenylsilyl) groups.

    Alternatively, R^1, R^2, $R^{2'}$, R^3 and/or $R^{3'}$ (formulae (I) or (III)), or R^5 or R^6 (formula (II)), can be chosen to

30   promote thermal cleavage of the bond linking the organic moiety to the non-bridging phosphate, phosphorothioate or

phosphoroselenoate oxygen. Cleavage of the bond linking
the organic moiety to the non-bridging phosphate,
phosphorothioate or phosphoroselenoate oxygen is
indicated by the dotted lines shown in Figs. 13A and 13B.

5   Thermal cleavage can be advantageous in that the use of
harsh chemicals, such as ammonium hydroxide, is avoided.
As such, thermal cleavage provides a mild alternative
that can be desirable for use in monomeric, oligomeric,
or polymeric compounds with chemically labile

10   substituents. It will be appreciated that certain
combinations or structural features of R^1, R^2, $R^{2'}$, R^3, and
$R^{3'}$, or of R^1, R^5, or R^6, can be chosen to promote thermal
cleavage of the bond linking the organic moiety to the
non-bridging phosphate, phosphorothioate or

15   phosphoroselenoate oxygen. For example, R^3 can be a
substituent that makes the bond linking the organic
moiety to the non-bridging phosphate, phosphorothioate or
phosphoroselenoate oxygen more labile, e.g., an electron
withdrawing group or a cation-stabilizing group, e.g., an

20   aryl, preferably a phenyl. Alternatively, R^3 and/or $R^{3'}$
can be a substituent that makes the carbon to which it is
attached less hindered (e.g., R^3 and $R^{3'}$ are H) and more
susceptible to internal thermal displacement by the C=X
residue.

25   An example of such a thermal cleavage is generally
illustrated in Fig. 13A. Specific examples of thermal
cleavage are shown in Fig. 13B. In one embodiment, R^1 is
an alkyl, R^2, $R^{2'}$, and $R^{3'}$ all are H, and R^3 is H or an
aryl. In a preferred combination, R^1 is methyl, $R^{2'}$ and $R^{3'}$

30   are H, and R^3 is H or phenyl. In a particularly
preferred combination, R^1 is methyl, and R^2, $R^{2'}$, R^3 and $R^{3'}$

all are H (e.g., as shown in Fig. 13B). When R^1 is
methyl, and R^2, R^3, $R^{2'}$ and $R^{3'}$ are H, thermal cleavage of
the bond linking the organic moiety to the non-bridging
phosphate, phosphorothioate or phosphoroselenoate oxygen

5   can be accomplished under fairly mild conditions. For
example, thermal cleavage in the two systems shown in
Fig. 13B (i.e., wherein X is O or S) can be carried out
to completion in about 80 minutes at about 80° C.

The present invention further provides a method of

10   preparing a polymer, including the steps of:

(a) reacting a nucleophile that can displace the N-
acyl group of an N-acylphosphoramidite of formula (I) or
(II), wherein R^4 is a protecting group with an N-
acylphosphoramidite of formula (I) or (II), preferably in

15   the presence of a base, to produce an adduct of the N-
acylphosphoramidite and the nucleophile, the adduct
comprising a tricoordinated phosphorus atom;

(b) reacting the adduct with a reagent selected from
the group consisting of oxidizing agents, sulfurizing

20   agents, and selenizing agents, to produce a product,
wherein the tricoordinated phosphorus atom is converted
into a phosphorus atom with a valence of greater than
three (e.g., a tetracoordinated phosphorous atom); and

(c) removing the protecting group R^4 from the

25   product.

Optionally, steps (a) through (c) can be repeated,
one or more times as necessary, until a polymer of
specified length is obtained.

Desirably, the method of the present invention

30   further comprises the step of the bond linking the
organic moiety to the non-bridging phosphate,

phosphorothioate or phosphoroselenoate oxygen atom (e.g.,
by aminolysis or thermal cleavage), after step (a), (b),
(c) or (d). While cleavage of the bond linking the
organic moiety to the non-bridging phosphate,

5 phoshphorothioate or phosphoroselenoate oxygen atom can
be done at any stage after any of steps (a)-(d), it is
preferably carried out after step (c) or (d). Most
preferably, the bond linking the organic moiety to the
non-bridging phosphate, phoshphorothioate or

10 phosphoroselenoate oxygen atom is cleaved thermally, for
example, as illustrated in Figs. 13A and 13B.

The N-acylphosphoramidite used in step (a) is a
compound of formula (I) or (II), wherein R^4 is a
protecting group. Preferably, the N-acylphosphoramidite

15 is a P-chiral N-acylphosphoramidite. When a P-chiral N-
acylphosphoramidite is used, the resulting adduct also is
P-chiral, since the coupling reaction (step (a)) occurs
with stereospecificity. Moreover, reaction of the
resulting adduct of step (a) with an oxidizing, a

20 sulfurizing, or a selenizing agent (step (b)) occurs
stereospecifically, that is, without any epimerization at
phosphorus. For example, sulfurization of the P-
diastereomerically pure adduct of step (a), obtained by
using a P-diastereomerically pure N-acylphosphoramidite,

25 results in a P-diastereomerically pure adduct. Although
sulfurization reactions are applied to adducts prepared
from standard phosphoramidite coupling chemistry, the
phosphorothioate products obtained thereby contain a
mixture of phosphorus stereoisomers (i.e., they are not

30 stereopure) because the phosphorus adducts prepared via
standard phosphoramidite chemistry contain a mixture of

stereoisomers. As indicated above, standard
phosphoramidite coupling reactions are not
stereospecific. Thus, the present invention
stereospecifically produces P-chiral coupling adducts

5   and, thus, provides access to oligonucleotides which are
stereochemically pure at phosphorus (e.g.,
oligonucleotide phosphorothioates).

Any suitable base can be used in coupling step (a)
including, for example, inorganic and organic bases.

10   Preferably, the base used in step (a) is a relatively
non-nucleophilic base, which is more preferably a
relatively non-nucleophilic amine base such as, for
example, tetramethylguanidine (TMG). Advantageously, and
preferably, the coupling conditions of the present

15   invention are carried out under basic conditions. As a
result, the use of an acid in the coupling reaction is
avoided, and the P-diastereomerically pure adduct formed
in step (a) does not epimerize. Since the coupling
reaction of step (a) occurs with complete

20   stereospecificity, the stereochemical purity with respect
to phosphorus can be governed by the stereochemical
purity of the N-acylphosphoramidite used therein.

Desirably, the method of the present invention
further includes the step of capping the unreacted

25   nucleophilic group after step (b) or (c). Capping is
usually done as a prophylactic measure to prevent the
unreacted nucleophilic groups, left over from prior
condensation reactions, from reacting in subsequent
condensation cycles. Capping promotes synthetic

30   advantages such as, for example, preventing the formation
of undesirable side products. When the nucleophile (or

oligomeric adduct, if steps (a)-(c) are repeated at least once) is a sugar hydroxyl, capping typically involves acylation of the unreacted sugar hydroxyls.

Typically, the reaction in step (a) leads to

5   formation of a tricoordinated P-chiral product, thereby enabling, in step (b), the formation of a P-chiral product. Deprotection of the preferred tetracoordinated P-chiral products can provide a P-chiral polymer of predetermined chirality and length. Preferably, the

10   nucleophile utilized in the method of the present invention is a nucleoside, an oligonucleotide, or a derivative thereof, step (a) utilizes a P-chiral N-acylphosphoramidite, and step (b) comprises sulfurization. Repeating the steps (a)-(c) can be

15   continued as many times as desired, until a polymer of a particular length and chirality is obtained.

As discussed above, formation of a tricoordinated P-chiral product in step (a) can be achieved by using any suitable P-chiral N-acylphosphoramidite, most preferably

20   a P-chiral analog of compound (I) or (II). In accordance with the present invention, P-chiral N-acylphosphoramidites can be obtained by any suitable method such as, for example, chiral synthesis, chromatographic resolution, or any suitable combination

25   thereof. Chromatographic separation of a mixture of P-chiral isomers can be facilitated, for example, if the monomeric subunit of the N-acylphosphoramidite is a chiral molecule, as illustrated, for example, in Scheme 2.

38

DMTr = dimethoxytrityl
R¹ and B are as defined herein
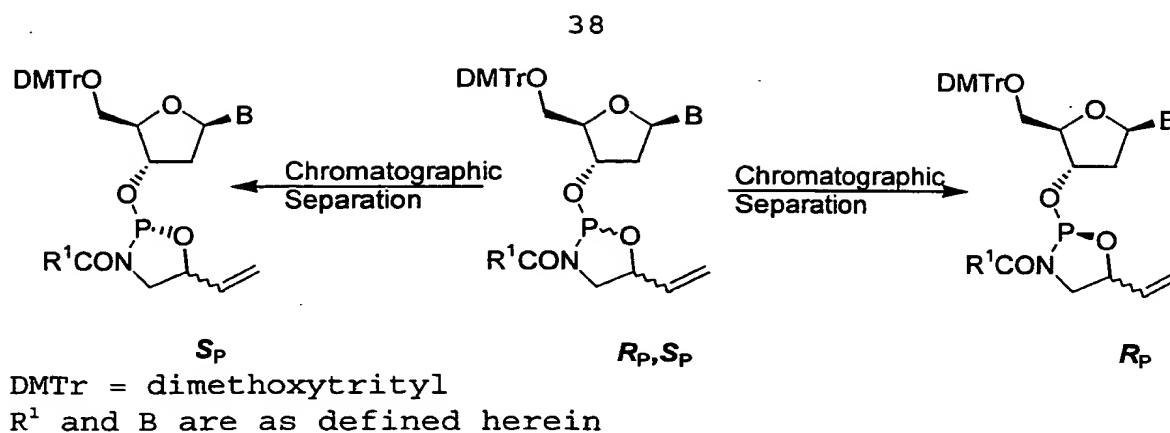
Scheme 2

5   Using this technique, P-chiral products having any

desired phosphorus stereochemistries can be

stereospecifically prepared simply by selecting the

appropriate P-chiral N-acylphosphoramidite and using it

10   in accordance with the method of the present invention.

The present invention provides new chemistry for

synthesizing oligonucleotides and related polymers having

phosphate or phosphate analogue linkages. In particular,

whenever phosphate analogue linkages are P-chiral, the

15   present invention provides a method for synthesizing

polymers having a predetermined sequence of P-chirality

along the polymer backbone. P-chiral oligonucleotides

obtained by the method of the present invention can be

employed as hybridization probes, therapeutic agents,

20   e.g., selective protein expression inhibitors, and the

like.

The methods and compounds of the present invention

offer other unique advantages, such as moisture

stability. In particular, the N-acylphosphoramidites of

25   the present invention are far more stable to moisture

under the coupling conditions of step (a) than are the

conventional phosphoramidite synthons for which mild acid

conditions are required. Moisture instability is a major
disadvantage inherent in oligonucleotide synthesis using
standard phosphoramidite chemistry. In particular,
standard phosphoramidite precursors hydrolytically

5 degrade, rapidly, upon contact with moisture under
standard (acidic) conditions which are required to
accomplish a coupling reaction. As such, acid-promoted
phosphoramidite nucleoside couplings typically are
carried out in a scrupulously moisture-free environment,

10 particularly if the target polymer comprises a large
number of monomeric units. The requirement of
maintaining an essentially water-free environment
dramatically increases the cost and complexity
oligonucleotide synthesis using standard phosphoramidite

15 chemistry. Since the N-acylphosphoramidites of the
present invention undergo hydrolytic degradation
sluggishly, or not at all, under the coupling conditions
of step (a), the problem of competitive hydrolytic
cleavage has essentially been eliminated. As such, the

20 method of the present invention need not be carried out
in a scrupulously water-free environment.

In a preferred embodiment, the nucleophile is
attached to a solid support. Accordingly, the present
invention provides a novel approach to solid phase

25 synthesis, in particular, the synthesis of
oligonucleotides and related polymers, using N-
acylphosphoramidites, for example, hydroxyl-protected
monomeric-O-(O-protected)-(N-acyl)phosphoramidites.

When the nucleophile is attached to a solid support,

30 the nucleophile is preferably a compound of the formula:

$$R^4 \text{---} O \text{---} Q \text{---} OH$$
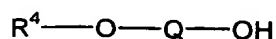
wherein Q is a nucleoside, an oligonucleotide comprising
a nucleoside, or an oligomer comprising a nucleoside,
wherein the nucleoside is of the formula:

5   wherein B and E are as defined herein, or an oligomer
which includes one of these nucleosides as a component
thereof, and R^4 is the solid support.

Desirably, the nucleophile is a monomer. In a
preferred embodiment, the nucleophile is a monomer and
10   is attached to a solid phase support through a linking
group that will resist cleavage in the presence of a
base, for example, a base used in step (a), thereby
allowing the resulting oligomer/polymer to remain
attached to the solid support throughout each successive
15   coupling step. When a solid support is used in
connection with a nucleophile (e.g., a nucleophilic
monomer), Q is preferably a nucleoside of the formula:
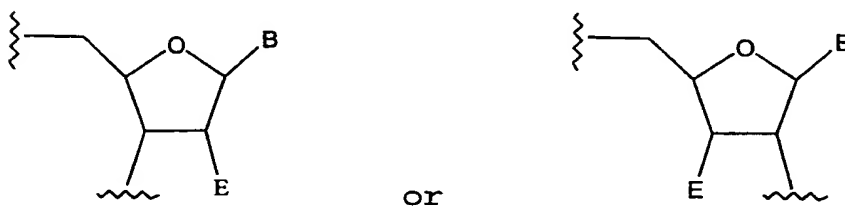
wherein B and E are as defined herein. In one preferred
20   embodiment, Q is a nucleoside substituent having a
defined stereochemistry, and is represented by the
formula:

or
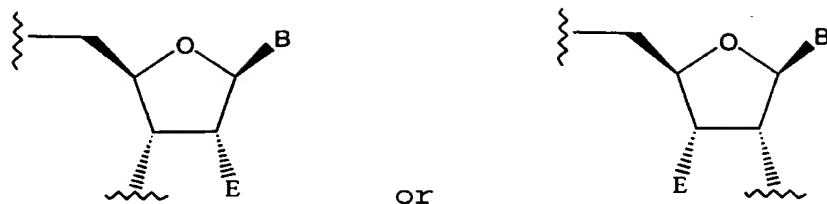
wherein B and E are as defined herein.

In a particularly preferred embodiment, a cyclic N-acylphosphoramidite of formula (I) is used to effect the

5   desired coupling, and is represented by the formula:

, or
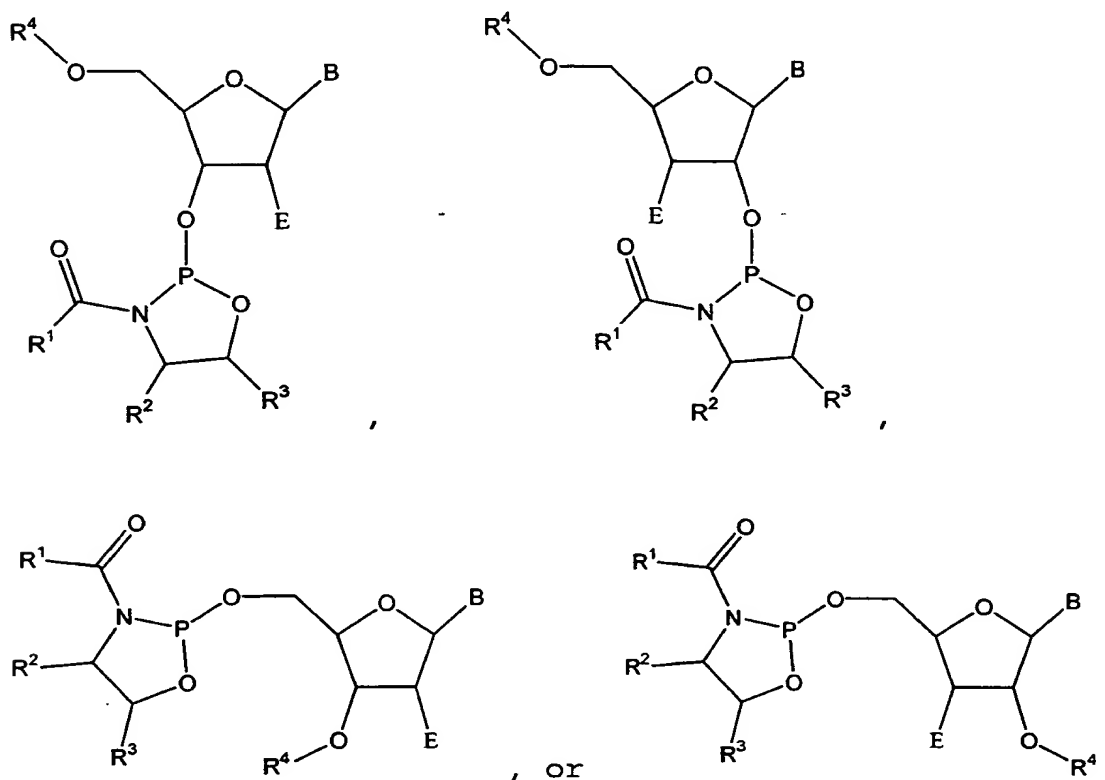
wherein R^1-R^4, B, and E are as defined herein.

10   Preferably, B is a purine, a pyrimidine, adenine, guanine, cytosine, uracil, or thymine, wherein B is unsubstituted or substituted with one or more substituents, which are the same or different, selected from the group consisting of a protecting group, R^{11}, OR^{11},

15   NHR^{11}, $NR^{11}R^{12}$, CN, NO_2, N_3, and a halogen, wherein R^{11} and R^{12} are as defined herein.

42

In one preferred embodiment, R^1 is an alkyl, which
is unsubstituted or substituted with one or more
substituents selected from the group consisting of
fluorine, OR^7 and SR^7, wherein R^7 is an alkyl or an aryl.

5   More preferably, R^1 is a C_1-C_6 alkyl, which is
unsubstituted or substituted with one or more fluorine
atoms. Still more preferably, R^1 is a methyl, which is
unsubstituted or substituted with one or more fluorine
atoms, and is most preferably fluoromethyl.

10   In another preferred embodiment, R^2, $R^{2'}$, R^3, or $R^{3'}$ is
a vinyl group, a phenyl or a benzyl. In still another
preferred embodiment, R^4 is a 4,4'-dimethoxytrityl group.

Oxidizing agents that can be used in the context of
the present invention include any suitable reagent that

15   can oxidize a tricoordinated phosphorus atom, particularly
a phosphite, to provide a phosphorus atom having a valence
of higher than three, preferably a tetracoordinated
phosphorus atom such as, for example, a phosphate, or an
equivalent thereof. Suitable oxidizing agents include,

20   for example, I_2/H_2O, peroxides, such as *tert*-
butylhydroperoxide, and the like.

Sulfurizing agents include any suitable reagent that
can sulfurize a tricoordinated phosphorus atom,
particularly a phosphite, to provide a phosphorus atom

25   with a valence of greater than three, preferably a
tetracoordinated phosphorus atom such as, for example, a
phosphorothioate, or an equivalent thereof. Suitable
sulfurizing agents include, for example, 3*H*-1,2-
benzodithiol-3-one 1,1-dioxide ("Beaucage Reagent"),

30   phenylacetyl disulfide, bis(*O*,*O*-
diisopropoxyphosphinothioyl) disulfide, and the like.

43

Selenizing agents include any suitable reagent that can selenize a tricoordinated phosphorus atom, particularly a phosphite, to provide a phosphorus atom having a valence of greater than three, preferably a

5   tetracoordinated phosphorus atom such as a phosphoroselenoate, or an equivalent thereof. Suitable selenizing agents include, for example, potassium selenocyanate (KSeCN) or elemental selenium.

The present invention also provides an alternative

10   method to the synthesis of unmodified oligonucleotides and to the non-stereospecific synthesis of oligonucleotide analogues. The alternative method of the present invention comprises:

(i) providing a nucleophile;

15   (ii) reacting the nucleophile, in the presence of a mild acid, with a synthon of the formula:
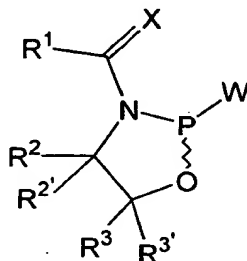
wherein X and R^1-$R^{3'}$ are as defined herein, and W is a leaving group amenable to nucleophilic displacement, to

20   produce an adduct of the nucleophile and the synthon, which is an N-acylphosphoramidite having a tricoordinated phosphorus atom;

(iii) reacting, in the presence of a base, the resulting adduct with a nucleoside, having at least one

25   nucleophilic group and at least one suitably protected nucleophilic group, to produce a product;

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(iv) deprotecting the protected nucleophilic group
of the resulting product;

(v) oxidatively transforming the tricoordinated
phosphorus atom into a tetracoordinated one; and

5   (vi) repeating the steps (ii)-(v) until an oligomer
or polymer of predetermined length is obtained.

Preferably, the method further comprises the step of
capping unreacted nucleophilic groups after step (iv) or
(v), as discussed herein. It is further preferred to

10   attach the first monomer (i.e., the nucleophile in the
first coupling reaction of a synthesis) to a solid phase
support through a linking group that will resist
cleavage, when in the presence of the base used in step
(iv).

15   It is preferred that W is a leaving group that can
be displaced by a monomer of the formula R^4-O-Q-OH or
R^4-O-Q^1-OH, wherein R^4, Q, and Q^1 are as defined herein.
In a preferred embodiment, W is halogen, a dialkylamino
having from 2 to about 8 carbon atoms (e.g.,

20   dimethylamino, diethylamino, N-methyl-N-isopropylamino,
and the like), or a cyclic amine substituent having from
2 to about 6 carbon atoms (e.g., pyrrolidinyl,
piperidinyl, morpholinyl, aziridinyl, and the like),
wherein one or more carbon atoms of the dialkylamino and

25   cyclic amine substituents are unsubstituted or
substituted with one or more heteroatoms, which are the
same or different. More preferably W is Cl,
dialkylamino, or a cyclic amino. Most preferably, W is
diethylamino.

30   The reactions in steps (iii) and (iv) enable the
formation of the tricoordinated P-chiral product and,

preferably, step (v) causes formation of the
tetracoordinated P-chiral product in a stereospecific
manner. Moreover, further deprotection preferably gives
either a P-achiral or a P-chiral polymer of predetermined

5   length. In step (iv), suitably protected nucleosides
comprise unmodified and/or modified nucleosides. Step (v)
preferably comprises oxidation and/or sulfurization.

In either method of the present invention, it is
preferred that an N-acylphosphoramidite of formula (I) is

10   used. Thus, in a preferred embodiment, the resulting
product of steps (a)-(c), (a)-(d), (iii), or (iii)-(v) is
a compound of formula (III). Compounds of formula (III)
are dimeric, when one coupling step is performed (n=1).
However, any desired number of subsequent coupling steps

15   can be performed, typically requiring deprotection (step
(c) or step (iv)) prior to subsequent coupling reactions,
wherein each monomeric unit defined by "n" is the same or
different, and the substituents R^1-R^4, R^{15}, X, Q^1, and Q are
as defined herein. Compounds of formula (III) are useful

20   in the synthesis of polymers, particularly phosphodiester-
linked polymers, more particularly P-chiral
phosphodiester-linked polymers, which can be obtained from
(III) via cleavage of the 2-amidoethoxy fragment (i.e.,
the bond linking the organic moiety to the non-bridging

25   phosphate, phosphorothioate or phosphoroselenoate oxygen
atom), as described herein.

Oligomers and polymers synthesized in accordance
with a preferred aspect of the present invention are
typically represented by the formula:

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$$R^4O-\left[\begin{array}{c} Q \\ \underset{O}{\big|} \\ O\diagdown P\diagup X \\ \underset{Y}{\big|}O \end{array}\right]_n Q-OH$$
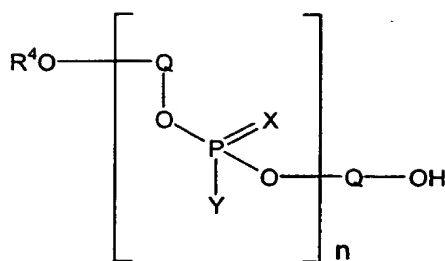
(IIIA)

wherein: Q, X, and n are as defined herein, and Y is any
suitable heteroatom or organic substituent, preferably

5   hydroxyl (or a suitable salt thereof). Preferably n is
in the range from about 3 to about 200; more preferably,
n is in the range from about 10 to about 40; and most
preferably in the range from about 15 to about 25. In
the polymers synthesized using the methods and compounds

10   of the present invention, Q, X, and Y, or any combination
thereof, can be the same or different when n is 1, and
can be the same or different in each of the units defined
by n when n is greater than 1.

     R^4 is preferably a hydrogen or a hydroxyl protecting

15   group such as, for example, a 4,4'-
dimethoxytriphenylmethyl (DMTr), 4-methoxy-
triphenylmethyl (MMTr), pixyl, acetyl, 9-
fluorenylmethyloxycarbonyl (Fmoc), t-butyldimethylsilyl
(TBDMS), and the like. Alternatively, R^4 is a reporter

20   group such as, for example, an amine, a mercapto, a
phosphate, a phosphorothioate, and the like. Reporter
groups preferably contain an active moiety for further
reaction with radioactive label such as, for example, [32]P-
phosphate, ^{125}I-iodinated Bolton-Hunter reagent, and the

25   like, or a non-radioactive label such as, for example,
fluorescein isothiocyanate (FITC), dansyl chloride, and
the like, or any other biologically active group such as,

for example, biotin, digoxigenin, and the like. Reporter
groups can be introduced by means known to those skilled
in the art including, for example, introduction of
appropriate linkers, spacers, arms, or other reagents
5   used for manipulating the distance between the reporter
group and the polymer.

X in formula (IIIA) is preferably S, O, or Se or a
substituted imino of the formula $=NR^{16}$, wherein R^{16} is an
alkyl, an aryl, or an alkenyl-substituted aryl
10   substituent. Preferably, Y is an OH (or suitable salt
thereof).

In a preferred embodiment, P-chiral polymers of the
present invention are of formula (IIIA) above, wherein X
and Y, or any combination thereof, can be the same or
15   different in any of the units being defined by n. More
preferably, P-chiral oligonucleotides prepared in
accordance with the present invention are of the formula:

48
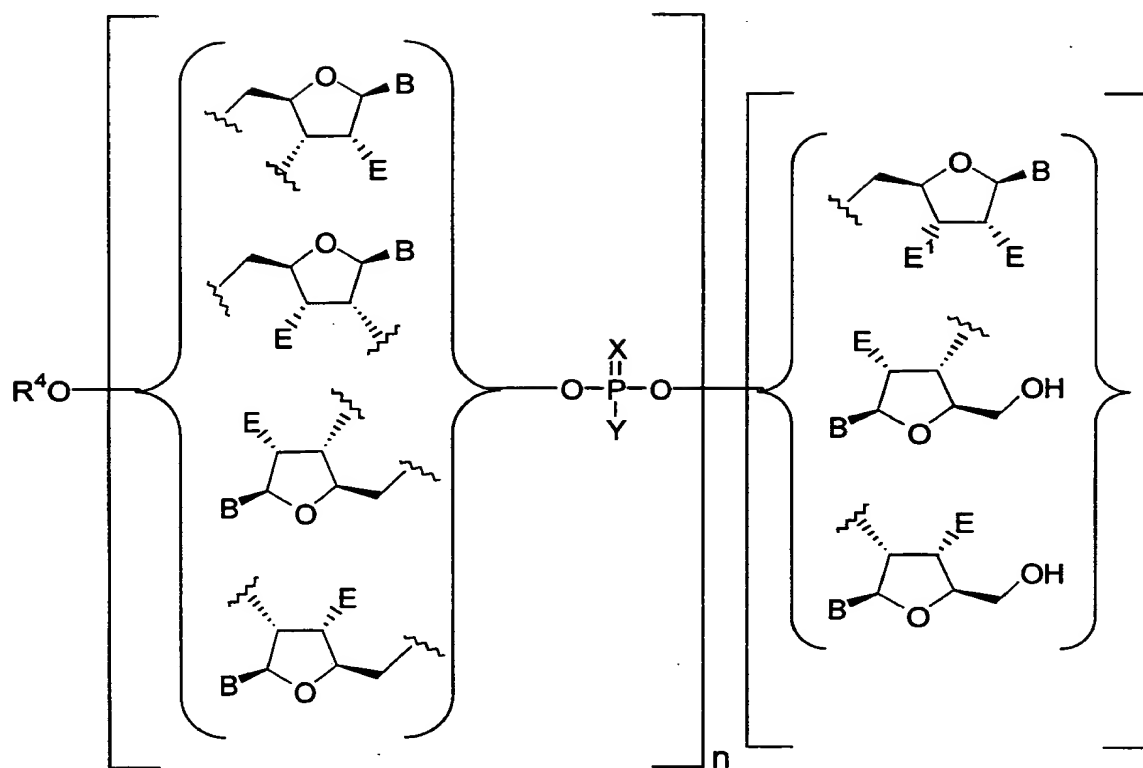
wherein X, Y, B, E and R⁴ are as defined herein, and E¹ includes the same groups defined herein with respect to E, and E and E¹ can be the same or different. B is

5   preferably a natural or a synthetically modified nucleic base, or B is a synthetic analog or reporter group, preferably a reporter group comprising a carboxyl, an alkyl, or an alkylamine. E¹ is preferably a 3'-hydroxyl (optionally protected), and E is preferably a hydrogen, a

10   halogen, a hydroxyl, or an appropriately protected hydroxyl, an amine, or an appropriately protected amine, or the like.

A polymer of any suitable length can be prepared in accordance with the method of the present invention.

15   Preferably, n is in the range from about 3 to about 200, but is more preferably in the range from about 12 to about 60. It is understood that the P-chiral oligonucleotides of the invention can include linkages,

49

for example, 5'-3', 5'-2', 5'-5', 3'-3', 2'-2', and 3'-2'
linkages, between nucleosides by the appropriate
selection of Q and Q^1, as defined herein.

5   The compounds of the present invention represented
by formulae (I) and (II) are typically prepared from a
synthon of the formula:

(IA)   or   (IIA),
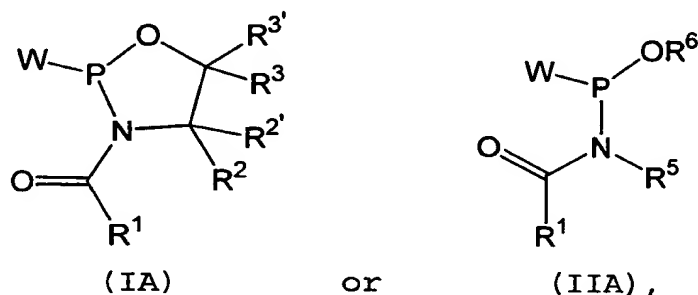
10   wherein R^1-$R^{3'}$, R^5, and R^6 are as defined herein, and W is
a leaving group amenable to nucleophilic attack by a free
group of the monomer, preferably a monomer of the formula
R^4-O-Q-OH or R^4-O-Q^1-OH, wherein R^4, Q, and Q^1 are as
defined herein. Preferably, W is halogen, a dialkylamino
15   having from 2 to about 8 carbon atoms, or a cyclic amine
substituent having from 2 to about 6 carbon atoms,
wherein at least one carbon of the alkyl groups in the
dialkylamino and cyclic amine substituents is optionally
substituted with one or more heteroatoms, which are the
20   same or different. More preferably W is Cl,
dialkylamino, or a cyclic amino. Most preferably, W is
diethylamino.

EXAMPLES

25   The following examples further illustrate the present
invention but, of course, should not be construed as in
any way limiting its scope.

Example 1

This example illustrates a general synthesis of the compounds of the present invention. The reaction schemes

5   referenced in this example are generally illustrated in Fig. 1.

Typically, the synthon precursor 5 (Fig. 1) is synthesized by first refluxing a mixture of acrolein (1), trimethylsilyl cyanide, and catalytic amounts of zinc

10   iodide according to the procedure reported by Gardrat et al. (J. Heterocyclic Chem. 1990, 27, 811). Reduction of the resulting nitrile 2 with LiAlH$_4$ in Et$_2$O afforded amino-alcohol 3. Heating 3 with a slight excess (1.1 molar equiv) of ethyl fluoroacetate at 120 °C until all

15   ethyl alcohol has distilled off gave the hydroxylated amide 4 in 88% yield (b.p. 83-84 °C/0.1 torr). An equimolar solution of hexaethylphosphorus triamide and 4 was heated to 120 °C until all diethylamine has distilled off. Vacuum distillation afforded the oxazaphospholane 5

20   in 69% yield.

Nucleoside cyclic acylphosphoramidite 7 was prepared by the reaction of a suitably protected nucleoside 6 with equimolar amounts of 5 and 1H-tetrazole in anhydrous dichloromethane for 4 h at ambient temperature.

25   Following evaporation of the reaction mixture, the residue is purified using a short silica gel column chromatography. The nucleosidic synthon 7 is rapidly eluted with a solution of acetonitrile:chloroform (1:2 v/v). Removal of the eluent under reduced pressure

30   afforded 7 as a white foam. The nucleoside cyclic

acylphosphoramidite **9** is prepared in a similar manner
from nucleoside **8** and compound **5**.

Example 2

5   This example illustrates a solid phase synthesis in
accordance with the present invention. The general
reaction scheme is illustrated in Fig. 2, in which
nucleoside cyclic acylphosphoramidite **7** (Fig. 1) is
specifically applied to the manual solid-phase synthesis

10   of a decanucleotide (dC_{10}). A solid support is denoted in
Figs. 2 and 3 by a darkened sphere with "S" in the
center.

       Because of the sensitivity of standard succinyl
linkers to strong bases, the first nucleoside monomer was

15   attached to long chain alkylamine controlled pore glass
(LCAA-CPG) to generate **10** has been modified. The
attachment of the leader nucleoside to LCAA-CPG is
accomplished *via* a sarcosine succinyl linkage according
to the method of Brown et al. (*J. Chem. Soc. Chem.*

20   *Commun.*, p. 891-893 (1989)). A column filled with 0.2
mmol of **10**, wherein the 5'-OH was protected with a DMTr
group, was treated with 2.5 mL of 3% trichloroacetic acid
in dichloromethane for 1 min to ensure complete cleavage
of the 5'-*O*-dimethoxytrityl (DMTr) protecting group. The

25   column was then washed with 5 mL of acetonitrile (MeCN)
and treated with a solution of **7** (10 mg) in 200 mL of
7.5% *N,N,N',N'*-tetramethylguanidine (TMG) in MeCN for 3
min. A solution (1 mL) of Cap A and Cap B (1:1) was
pushed through the column, left for 1 min, and then

30   washed with MeCN (5 mL), after which a solution of 1 M
tert-butylhydroperoxide in dichloromethane (1 mL) was

pushed through the column for 1 min, and washed with MeCN
(5 mL). This cycle was repeated 8 additional times.

5   Stepwise DMTr analysis indicated that each coupling
yield proceeded with high efficiency, typically 90% or
greater. The content of the column was then transferred
into a glass vial, and treated with concentrated ammonium
hydroxide for 10 h at 55 °C. The crude oligomer was
characterized by reversed phase (RP) HPLC and
polyacrylamide gel electrophoresis (PAGE). Both

10   techniques indicated that dC_{10} was prepared in a yield
consistent with that determined by the stepwise DMTr
analysis. In addition, crude dC_{10} was cleanly hydrolysed
by snake venom phosphodiesterase and alkaline phosphatase
to 2'-deoxycytidine with no evidence of either partially

15   deprotected nucleotides or nucleobase modifications.
To enable, for example, the synthesis of thioated
oligonucleotides stereogenically at phosphorus, the
synthon 7 (Fig. 1) must first be separated into its Rp
and Sp diasteroisomers (see Fig. 3). This is

20   accomplished by chromatography on functionalized silica
(C-1, C-2, C-4, C-8, or C-18 reversed-phase silica).

Example 3

This example illustrates the application of the
25   synthetic "cycle" described in Example 2, in the
stereospecific synthesis of oligonucleotide
phosphorothioates. The reaction scheme is illustrated
generally in Fig. 3.

A diastereomeric mixture of nucleosidic N-
30   acylphosphoramidite 7 was chromatographically separated
into its Rp and Sp isomers **7Rp** and **7Sp**, respectively.

53

Each P-chiral isomer was coupled with nucleophilic

monomer **10** (Fig. 2), using the conditions of Example 2,

to provide P-chiral adducts. The coupling reactions are

stereospecific. Sulfurization of the resulting adducts

5 results in the formation of the **11Sp** and **11Rp** isomers, as

illustrated in Fig. 3. Deprotection of the solid support

and the 2-amidoethoxy fragment from the sulfurized

products is therefore expected to provide

stereochemically pure Rp and Sp oligonucleotide products.

10   It should be noted that the oxidant in the oxidation

step is replaced by a sulfur-transfer reagent such as $3H$-

1,2-benzodithiol-3-one 1,1-dioxide, phenylacetyl

disulfide, bis(O,O-diisopropoxyphosphinothioyl)

disulfide, and the like. In order to ensure optimum

15 sulfurization, a capping step should be performed after

the sulfur transfer step.

Example 4

       This example illustrates the preparation of various

20 nucleosidic N-acylphosphoramidites of the present

invention, wherein the N-acyloxazaphospholane moiety is

introduced at different hydroxyls of a differentially

protected nucleoside core. The reaction schemes are

illustrated generally in Fig. 4.

25   Using the procedure of Example 1, nucleophilic

monomers **12**, **14**, **16**, and **18** were coupled to synthon **5**

using tetrazole, to provide nucleosidic N-

acylphosphoramidites **13**, **15**, **17**, and **19**, respectively.

The resulting nucleosidic N-acylphosphoramidites can be

30 used as a vehicle for one or more coupling reactions, to

provide oligomer or polymer products. Alternatively, the

resulting nucleosidic N-acylphosphoramidites can be
separated into their Rp and Sp isomers prior to their use
as coupling reagents. The pholane moiety of
nucleosidic N-acylphosphoramidites **13**, **15**, **17**, and **19** are
5   attached to either the 3'- or 5'- hydroxyl in the case of
2'-deoxyribonucleosides or, additionally, to the 2'-
hydroxyl in the case of ribonucleosides. These products
also represent various ribonucleoside monomers that can
be used for solid-phase synthesis (both stereospecific
10 and non-stereospecific) of oligoribonucleotides and their
analogues as illustrated in Fig. 2 and Fig. 3.

Example 5

    This example illustrates the preparation of acyclic
15 N-acylphosphoramidites, and methods of using them in the
context of the present invention. The nucleoside
acylphosphoramidites can be applied in a manner similar
to that described in Examples 2 and 3, and Figs. 2 and
Fig. 3. The reaction scheme is illustrated generally in
20 Fig. 5. A solid support is denoted in Fig. 5 by a
darkened sphere with "S" in the center.

    As illustrated in Fig. 5, the non-nucleosidic
chlorophosphoramidite derivative **20** is condensed with a
suitable *N*-methylamide (**21**) to generate the
25 acylphosphoramidite **22**. Reaction of **22** with suitably
protected nucleosides **6** (Fig. 1) in the presence of 1*H*-
tetrazole affords the corresponding nucleoside 3'-
acylphosphoramidites **23** as a mixture of P-
diastereoisomers. These amidites are activated under
30 basic conditions and are expected to be useful in solid-
phase oligonucleotide synthesis in a manner similar to

that shown in Fig. 2. Nucleoside 5'-acylphosphoramidites

similar to **9** (Fig. 1) also can be applied for the same

purpose. Alternatively, separation of the *Rp*- and *Sp*-

diastereoisomers of **23** are expected to enable the

stereospecic synthesis of thioated oligonucleotides in a

manner similar to that illustrated in Fig. 3. In this

context, ribonucleoside acylphosphoramidites of formula
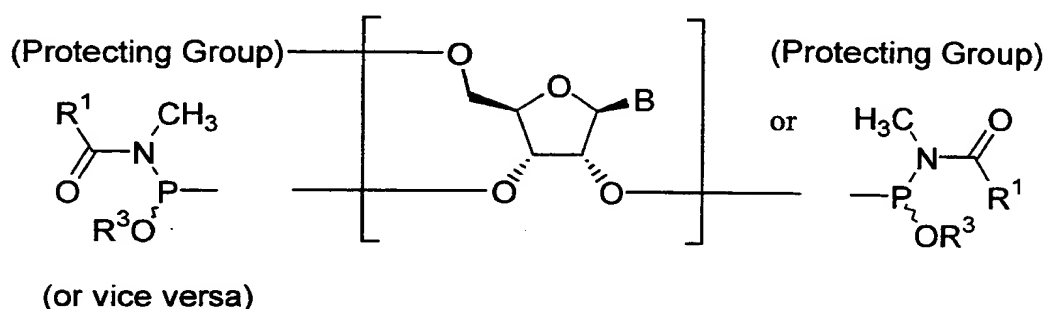
can be used in accordance with the present invention for

ribonucleotide syntheses, and are expected to work in the

same manner as the cyclic species, for example, **13**, **15**,

17, and **19** (Fig. 4).

Example 6

        This example demonstrates an alternate approach to

the synthesis of oligonucleotides *via* nucleoside cyclic

acylphosphoramidites and acylphosphoramidites, as

illustrated in Figs. 6 and 7. A solid support is denoted

in Figs. 6 and 7 by a darkened sphere with "S" in the

center. The strategy was demonstrated by reacting non-

nucleosidic cyclic N-acylphosphoramidite **5** (Fig. 1) and

acylphosphoramidite **22** (Fig. 5) with the functionalized

solid-support-bound **10** (Fig. 2) in the presence of 1H-

tetrazole to generate **25** and **26**, respectively, as shown

56

in Fig. 6. The reaction of suitably protected nucleoside

6 with **25**, or **6** with **26**, under basic conditions, followed

by oxidation, provided dinucleotides **11** and **27**,

respectively (Scheme 7). Deprotection of **11** and **27**

5   provides the same dinucleotide, as shown in Fig. 7. The

same strategy applies with respect to the synthesis of

ribonucleotide and the non-stereospecific synthesis of

thioated oligonucleotides. The solid-phase synthesis of

a decanucleotide (dC_{10}) has been achieved using a DNA

10   synthesizer.

General Protocol for Examples 7-12

        For the synthesis of oligonucleotides using 5'-*O*-

dimethoxytrityl-3'-*O*-(5-phenyl-3-N-fluoroacetyl)-1,3,2-

15   oxazaphospholanyl-2'-*O*-deoxyribonucleoside derivatives in

examples 7-12, the general protocol is as follows. The

syntheses were performed in a standard DNA synthesis

column as available from many suppliers. Standard LCAA-

CPG from Applied Biosystems (Masterpiece) columns were

20   used.

        The syntheses were carried out by way of the

following general steps. The steps were not necessarily

done in numerical order within a particular synthesis

cycle. The particular sequence of steps used is

25   indicated separately in each example.

        In step 1, the appropriate CPG-bound nucleoside is

detritylated in accordance with a standard procedure.

        In step 2, 5'-*O*-Dimethoxytrityl-3'-*O*-(5-phenyl-3-*N*-

fluoroacetyl)-1,3,2-oxazaphospholanyl-2'-*O*-

30   deoxyribonucleoside derivatives (5 mg, ca. 5 μmol) are

dissolved in acetonitrile (200 μL). Tetramethylguanidine

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(TMG, 4 μl, ca. 30 μmol) is subsequently added and the mixture is applied to the synthesis column.

In step 3, a standard oxidation or sulfurization reaction is carried out after the reaction of step 2 is continued for 5 min.

Steps 2 and 3 are repeated to optimize the yield for a particular synthesis cycle. Steps 2 and 3 need not be performed more than once for a particular synthesis cycle. However, yields are typically improved (e.g., resulting in nearly 100% overall yield) if steps 2 and 3 are repeated within a particular synthesis cycle. Optionally, steps 2 and 3 can be repeated three or more times, as desired, to optimize the yield for a particular synthesis cycle even further.

In step 4, the synthesis cycle is concluded with a capping step. Synthesis cycles can be repeated until the designed sequence length is obtained.

In step 5, the synthetic oligonucleotide is subjected to post-synthesis cleavage from the support, and deprotection.

Example 7

This example describes the synthesis of a dinucleotide, particularly $T_{po}T$. The following steps were used in the present example.

Step 1: The bound nucleoside was treated with 3% trichloroacetic acid/dichloromethane (Applied Biosystems DNA synthesis reagent (3 mL, 1 min)), followed by washing with acetonitrile (3 mL, 30s).

Step 2: 5'-*O*-Dimethoxytrityl-3'-*O*-(5-phenyl-3-*N*-fluoroacetyl)-1,3,2-oxazaphospholanyl-2'-*O*-deoxythymidine

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(5 mg, ca. 5 μmol) and tetramethylguanidine (TMG, 4 μl, ca. 30 μmol) in acetonitrile (200 μl) were added to the column and reacted for 5 min., followed by washing with acetonitrile (3 mL, 30s).

5   Step 3: The resulting product was treated with iodine/water/pyridine/tetrahydrofuran (Applied Biosystems DNA synthesis reagent), (500 μl, 30s), followed by washing with acetonitrile (3 ml, 30s).

        Step 5: The dinucleotide was cleaved from the
10   support by treatment with concentrated aqueous ammonium hydroxide solution (1 mL, 10h, 55°C).

        In the present example, a standard column DMT-T-LCAA-CPG (0.2 μmol) was used and was subjected to the above steps in the following sequence:

15

                    1, 2, 3, 2, 3, 1, 5

        The ammoniacal solution obtained in the final step was concentrated under reduced pressure and analyzed by
20   RP-HPLC.

Example 8

        This example describes the synthesis of P-diasteriomerically pure phosphorothioate $[R_p]$-$C_{PS}C$. The
25   following steps were used in the present example.

        Step 1: The bound nucleoside was treated with 3% trichloroacetic acid/dichloromethane (Applied Biosystems DNA synthesis reagent), (3 mL, 1 min), followed by washing with acetonitrile (3 mL, 30s).

30   Step 2: $[S_p]$-N^4-Benzoyl-5'-O-dimethoxytrityl-3'-O-(5-phenyl-3-N-fluoroacetyl)-1,3,2-oxazaphospholanyl-2'-O-

deoxycytidine (5 mg, ca. 5 μmol) and tetramethylguanidine
(TMG, 4μl, ca 30 μmol) in acetonitrile (200 μl) were
added to the column and reacted for 5 min., followed by
washing with acetonitrile (3 mL, 30s).

5   Step 3: The resulting product was treated with 3H-
1,2-benzodithiol-3-one 1,1-dioxide (1% Beaucage Reagent
in acetonitirile (w/v)), 3 min., followed by washing with
acetonitirile (3 mL, 30s).

       Step 5: The dinucleotide was cleaved from the
10   support by treatment with concentrated aqueous ammonium
hydroxide solution (1 mL, 10 h, 55°C).

       In the present example, a standard column DMT-C^{Bz}-
LCAA-CPG (0.2 μmol) was used and was subjected to the
above steps in the following sequence:

15

1, 2, 3, 2, 3, 1, 5

       The ammoniacal solution obtained in the final step
was concentrated under reduced pressure and analyzed by
20   RP-HPLC.

Example 9

       This example describes the synthesis of a P-
diastereomerically pure phosphorothioate-linked
25   trinucleotide (trimer), [R$_p$,R$_p$]C$_{PS}$C$_{PS}$C. The following
steps were used in the present example.

       Step 1: The bound nucleoside was treated with 3%
trichloroacetic acid/dichloromethane (Applied Biosystems
DNA synthesis reagent), (3 mL, 1 min), followed by
30   washing with acetonitirile (3 mL, 30s).

       Step 2:   [Sp]-N^4-Benzoyl-5'-O-dimethoxytrityl-3'-O-

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(5-phenyl-*N*-fluoroacetyl)-1,3,2-oxazaphospholanyl-2'-*O*-

deoxycytidine (Fig. 8B, 5 mg, ca. 5 μmol) and

tetramethylguanidine (TMG,4 μl, oxazaphospholanyl-2'-*O*-

(5-phenyl-3-*N*-fluoroacetyl)-1,3,2-oxazaphospholanyl-2'-*O*-

5   deoxycytidine (5 mg, ca. 5 μmol) and tetramethylguanidine

(TMG, 4 μl, ca. 30 μmol) in acetonitrile (200 μl) were

added to the column and reacted for 5 min., followed by

washing with acetonitrile (3 mL, 30s).

Step 3: The resulting product from step 2 was

10   treated with 3*H*-1,2,-benzodithiol-3-one 1,1-dioxide (1%

Beaucage Reagent in acetonitrile (w/v)), 3 min., followed

by washing with acetonitrile (3 mL, 30s).

Step 4: The resulting product from step 3 was capped

with acetic anhydride/lutidine/tetrahydrofuran

15   (Applied Biosystems DNA synthesis reagent), (1 mL), mixed

with 1-methylimidazole/tetrahydrofuran (Applied

Biosystems DNA synthesis reagent), (1 mL), 2 min.,

followed by washing with acetonitrile (3 mL, 30s).

Step 5: The trinucleotide was cleaved from the

20   support by treatment with concentrated aqueous ammonium

hydroxide solution (1 mL, 10 h, 55°C).

In the present example, a standard column DMT-C^{Bz}-

LCAA-CPG (0.2 μmol) was used and was subjected to the

above steps in the following sequence:

25

1, 2, 3, 2, 3, 4, 1, 2, 3, 2, 3, 5

The ammoniacal solution obtained in the final step

was concentrated under reduced pressure to provide the P-

30   diastereomerically pure phosphorothioate-linked

trinucleotide (trimer), [R_P,R_P] $C_{PS}C_{PS}C$. The product

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obtained in accordance with the present invention was
analyzed by RP-HPLC (Fig. 10D). The trimer $C_{ps}C_{ps}C$ also
was prepared using a standard phosphoramidite coupling
reagent (Fig. 8A). The HPLC of $C_{ps}C_{ps}C$ obtained using the
5   standard phosphoramidite coupling reagent is shown in
Fig. 10A and contained, as expected, a mixture of all
four possible P-diastereomers (indicated by RR, SR, RS,
and SS). By contrast, the trimer prepared in accordance
with the present invention produced only one P-
10   diastereomer (RR). No other P-diastereomers were present
by HPLC in the product obtained in accordance with the
present invention, even in trace amounts. Co-injection
of the trimer prepared in accordance with the present
invention and the P-diastereomeric mixture obtained by
15   the standard phosphoramidite method (Fig. 10E) confirmed
that the product obtained in accordance with the present
invention was indeed $[R_p,R_p]C_{ps}C_{ps}C$. This example
demonstrates that the N-acylphosphoramidites of the
present invention produce oligomers that are P-
20   diastereomerically pure.

Example 10
      This example describes the synthesis of a P-
diastereomerically pure phosphorothioate-linked
25   trinucleotide (trimer), $[S_p,S_p]C_{ps}C_{ps}C$. The following
steps were used in the present example.
      Step 1: The bound nucleoside was treated with 3%
trichloroacetic acid/dichloromethane (Applied Biosystems
DNA synthesis reagent), (3 mL, 1 min.), followed by
30   washing with acetonitrile (3 mL, 30s).

Step 2: [Rp]-*N*⁴-Benzoyl-5'-*O*-dimethoxytrityl-3'-*O*-(5-phenyl-3-*N*-fluoroacetyl)-1,3,2-oxazaphospholanyl-2'-*O*-deoxycytidine (Fig. 8C, 5 mg, ca. 5 μmol) and tetramethylguanidine (TMG, 4 μl, ca. 30 μmol) in

5   acetonitrile (200 μl) were added to the column and reacted for 5 min., followed by washing with acetonitrile (3 mL, 30s).

Step 3: The resulting product from step 2 was treated with 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (1%

10   Beaucage Reagent in acetonitrile (w/v)), 3 min., followed by washing with acetonitrile (3 mL, 30s).

Step 4: The resulting product from step 3 was capped with acetic anhydride/lutidine/tetrahydrofuran (Applied Biosystems DNA synthesis reagent), (1 mL), 2 min.,

15   followed by washing with acetonitrile (3 mL, 30s).

Step 5: The trinucleotide was cleaved from the support by treatment with concentrated aqueous ammonium hydroxide solution (1 mL, 10 h, 55°C).

In the present example, a standard column DMT-C^{Bz}-

20   LCAA-CPG (0.2 μmol) was used and was subjected to the above steps in the following sequence:

1, 2, 3, 2, 3, 4, 1, 2, 3, 2, 3, 5

25   The ammoniacal solution obtained in the final step was concentrated under reduced pressure and analyzed by RP-HPLC. The product obtained in the present example was analyzed by RP-HPLC (Fig. 10B). The trimer $C_{ps}C_{ps}C$ also was prepared using a standard phosphoramidite coupling

30   reagent (Fig. 8A). The HPLC of the $C_{ps}C_{ps}C$ obtained using the standard phosphoramidite coupling reagent is shown in

Fig. 10A and contained, as expected, a mixture of all
four possible P-diastereomers (indicated by RR, SR, RS,
and SS). By contrast, the trimer prepared in accordance
with the present invention produced only one P-

5   diastereomer (SS). No other P-diastereomers were present
by HPLC in the product obtained in accordance with the
present invention, even in trace amounts. Co-injection
of the trimer prepared in accordance with the present
invention and the P-diastereomeric mixture obtained by

10   the standard phosphoramidite method (Fig. 10C) confirmed
that the product obtained in accordance with the present
invention was indeed [S_P, S_P] $C_{PS}C_{PS}C$. This example further
demonstrates that the N-acylphosphoramidites of the
present invention produce oligomers that are P-

15   diastereomerically pure.

Example 11

      This example describes the synthesis of a P-
diastereomerically pure phosphorothioate-linked tetramer

20   [Rp, Sp, Rp] -$C_{PS}C_{PS}C_{PS}C$. The following steps were used in the
present example.

      Step 1: The bound nucleoside was treated with 3%
trichloroacetic acid/dichloromethane (Applied Biosystems
DNA synthesis reagent), (3 mL, 1 min.), followed by

25   washing with acetonitrile (3 mL, 30s).

      Step 2: [S_p]-N^4-benzoyl-5'-O-dimethoxytrityl-3'-O-(5-
phenyl-3-N-fluoroacetyl)-1,3,2,-oxazaphospholanyl-2'-O-
deoxycytidine (5 mg, ca. 5 μmol) and tetramethylguanidine
(TMG, 4 μl, ca. 30 μmol) in acetonitrile (200 μl) were

30   added to the column and reacted for 5 min., followed by
washing with acetonitrile (3 mL, 30s).

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Step 2': [Rp]-N^4-Benzoyl-5'-O-dimethoxytrityl-3'-O-(5-phenyl-3-N-fluoroacetyl)-1,3,2-oxazaphospholanyl-2'-O-deoxycytidine (Fig. 8C, 5 mg, ca. 5 μmol) and tetramethylguanidine (TMG, 4 μl, ca. 30 μmol) in

5   acetonitrile (200 μl)) were added to the column and reacted for 5 min., followed by washing with acetonitrile (3 mL, 30s).

Step 3: The resulting product from step 2 or 2' was treated with 3H-1,2-benzodithiol-3-one 1,1-dioxide (1%

10   Beaucage Reagent in acetonitrile (w/v)), 3 min., followed by washing with acetonitrile (3 mL, 30s).

Step 4: The resulting product from step 3 was capped with acetic anhydride/lutidine/tetrahydrofuran (Applied Biosystems DNA synthesis reagent), (1 mL), mixed with 1-

15   methylimidazole/tetrahydrofuran (Applied Biosystems DNA synthesis reagent), (1 mL), 2 min., followed by washing with acetonitrile (3 mL, 30s).

Step 5: The trinucleotide was cleaved from the support by treatment with concentrated aqueous ammonium

20   hydroxide solution (1 mL, 10 h, 55 °C).

In the present example, a standard column DMT-C^{Bz}-LCAA-CPG (0.2 μmol) was used and was subjected to the above steps in the following sequence:

25   1, 2, 3, 2, 3, 4, 1, 2', 3, 2', 3, 4, 1, 2, 3, 2, 3, 5

The ammoniacal solution obtained in the final step was concentrated under reduced pressure and analyzed by RP-HPLC. The product obtained in the present example was

30   analyzed by RP-HPLC (Fig. 11B).

The tetramer $C_{ps}C_{ps}C_{ps}C$ also was prepared using a
standard phosphoramidite coupling reagent (Fig. 8A). The
HPLC of the $C_{ps}C_{ps}C_{ps}C$ obtained using the standard
phosphoramidite coupling reagent is shown in Fig. 11A and

5   contained, as expected, a mixture of all eight possible
P-diastereomers (indicated by RRR, SRR, RRS, SRS, RSR,
SSR, RSS, and SSS). By contrast, the tetramer prepared
in accordance with the present example produced only one
P-diastereomer (RSR). No other P-diastereomers were

10 present by HPLC, even in trace a amounts. Co-injection
of the tetramer prepared in the accordance with the
present invention and the P-diastereomeric mixture
obtained by the standard phosphoramidite method (Fig.
11C) confirmed that the product obtained in accordance

15 with the present invention was indeed [Rp,Sp,Rp]-$C_{ps}C_{ps}C_{ps}C$.
This example further demonstrates that the N-
acylphosphoramidites of the present invention can
predictably produce oligomers that are P-
diastereomerically pure.

20

Example 12

   This example describes the synthesis of a P-
diastereomerically pure phosphorothioate-linked
undecamer, [all R_p]-$(T_{ps})_{11}T$ (eleven nucleoside units in

25 the oligonucleotide chain). The following steps were
used in the present example.

   Step 1: The bound nucleoside was treated with 3%
trichloroacetic acid/dichloromethane (Applied Biosystems
DNA synthesis reagent), (3 mL,. 1 min.), followed by

30 washing with acetonitrile (3 mL, 30s).

Step 2: [S$_p$]-5'-O-Dimethoxytrityl-3'-O-(5-phenyl-3-N-fluoroacetyl)-1,3,2-oxazaphospholanyl-2'-O-deoxythymidine (5 mg, ca. 5 μmol) and tetramethylguanidine (TMG, 4 μl, ca. 30 μmol) in

5   acetonitrile (200 μl) were added to the column and reacted for 5 min., followed by washing with acetonitrile (3 mL, 30s).

Step 3: The resulting product from step 2 was treated with 3H-1,2-benzodithiol-3-one 1,1-dioxide (1%

10   Beaucage Reagent in acetonitrile (w/v)), 3 min., followed by washing with acetonitrile (3 mL, 30s).

Step 4: The resulting product from step 3 was capped with acetic anhydride/lutidine/tetrahydrofuran (Applied Biosystems DNA synthesis reagent), (1 mL), 2 min.,

15   followed by washing with acetonitrile (3 mL, 30s).

Step 5: The trinucleotide was cleaved from the support by treatment with concentrated aqueous ammonium hydroxide solution (1 mL, 10 h, 55°C).

In the present example, a standard column DMT-T-

20   LCAA-CPG (0.2 μmol) was used and was subjected to the above steps in the following sequence:

[1, 2, 3, 2, 3, 4]$_{11}$, 5

25   The ammoniacal solution obtained in the final step was concentrated under reduced pressure and analyzed by RP-HPLC. The product obtained in the present example exhibited only one peak by HPLC. Although the HPLC system used in this example cannot chromatographically

30   separate all possible P-diastereomers for an oligomer of

this length, it is believed that the product obtained in the present example is indeed P-diastereomerically pure.

Example 13

5   This example demonstrates the hydrolytic stability of the N-acylphosphoramidites of the present invention (Fig. 9B), relative to the hydrolytic stability of standard phosphoramidites (Fig. 9A). The hydrolytic stability for each type of reagent was determined under

10   reaction conditions normally employed for each type of coupling reagent.

Samples of the dinucleotide $d(T_{po}G)$ were prepared by a standard coupling method using the phosphoramidite of Fig. 9A. Samples of $d(T_{po}G)$ also were prepared by a

15   coupling reaction in accordance with the present invention using the N-acylphosphoramidite of Fig. 9B. Each coupling method was performed in the absence of moisture and in the presence of moisture (0.1% water).

The products were analyzed by HPLC. The HPLC

20   chromatogram of the product obtained via the standard phosphoramidite reagent (Fig. 9A) in a moisture-free environment is shown in Fig. 12A. The HPLC chromatogram of the product obtained using the standard phosphoramidite of Fig. 9A in the presence of 0.1%

25   moisture is shown in Fig. 12B. The HPLC chromatogram of the product obtained via the N-acylphosphoramidite (Fig. 9B) in a moisture-free environment is shown in Fig. 12C. The product obtained using the N-acylphosphoramidite of Fig. 9B in the presence of 0.1% moisture is shown in Fig.

30   12D.

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The target product is indicated in the HPLC

chromatogram by a peak labeled d(T$_{po}$G), corresponding to

the dinucleotide. Hydrolysis of the coupling reagent

(i.e., hydrolytic instability) is indicated in the HPLC

5   chromatogram by the presence of a peak corresponding to

the single nucleoside, indicated by dG.

The HPLC's confirmed that the same product (d(T$_{po}$G))

was obtained by either method when the reactions were

carried out in a moisture-free environment (Figs. 12A and

10   12C). However, when the same reactions were carried out

in the presence of moisture, the product obtained by the

standard phosphoramidite method contained only a trace of

the desired product, and was almost entirely the

uncoupled single nucleoside dG (Fig. 12B). Thus, as

15   expected, the standard phosphoramidite was hydrolytically

unstable under coupling conditions in which moisture was

present. By contrast, the product obtained using the N-

acylphosphoramidite (Fig. 9B) contained mostly the

desired product, and a relatively minor amount of the

20   uncoupled single nucleoside dG, even when the coupling

reaction was performed in the presence of significant

moisture (Fig. 12D). These results demonstrate that the

N-acylphosphoramidites of the present invention are

hydrolytically stable, even in the presence of a

25   significant amount of moisture, under coupling conditions

used in connection with such compounds.

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All of the references cited herein, including patents, patent applications, and publications, are hereby

5   incorporated in their entireties by reference.

While this invention has been described with an emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that variations of the preferred embodiments may be used and that it is intended

10   that the invention may be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the invention as defined by the following claims.